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THE COMPETENCE OF MANDIBULAR ARCH ECTOMESECHYME IN 9 to 11 DAY EMBRYO MICE

by

LINDA KATHLEEN BOURGEOIS,B.SC.,D.D.S.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE COMPETENCE OF MANDIBULAR ECTOMESECHYME IN 9 to 11 DAY EMBRYO MICE submitted by LINDA KATHLEEN BOURGEOIS,B.SC.,D.D.S. in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

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Date.....23/4/85.....

ABSTRACT

The Competence of Mandibular Arch Ectomesenchyme in Embryo Mice.

Each quadrant of a mammalian dentition contains three classes of teeth: incisors, canines and molars. A review of the scientific literature reveals several theories of tooth development, each attempting to explain the development of tooth primordia in this distinct pattern.

Butler (1939) postulated the existence of three separate morphogenetic fields wherein a chemodifferentiator influences a predetermined number of identical tooth primordia alréady present in the developing jaw to form teeth of the appropriate size, shape and position for that species. Wolpert (1978) suggests a positional information theory whereby cells receive information regarding their position relative to the field boundaries. Although Wolpert describes chick limb bud development to support his theory, the concept can be applied to tooth development as well. Embryonic mandibular arch cells would interpret positional cues and differentiate accordingly to form the appropriate pattern of tissues. Extrinsic control of tooth development and pattern formation is the dominant feature of Butler and Wolpert's theories.

Osborn (1978a) has postulated that the teeth in each class sequentially unfold from incisor, canine and molar clones of cells present in the embryonic jaw arches, without the need for extrinsic controls such as morphogenetic substances or field generators. The following study accepts the hypothesis that clones may exist and attempts to identify them.

Mouse mandibular arch tissue (9 to 11 day fetuses) was surgically removed and cut into anterior, middle and posterior segments. These were cultured in the anterior eye chamber of homologous hosts to determine their capacities to differentiate dental tissues, in the absence of normal environmental cues. Tissues from the middle segment of 9 or 10 day mandibular arches were capable of forming molar teeth with their supporting tissues. The anterior and posterior segments produced other tissues, but not teeth. At 11 days two segments produced teeth. The anterior segment produced an incisor and the middle or posterior segment produced a molar (or molars). This is equivalent to the adult state because canines are not developed in mice and in

terms of the clone hypothesis the canine clone is replaced by a toothless region, the diastema.

Data gathered from this series of experiments can be interpreted to support the clonal theory of tooth development. The group of cells present in the middle segment of 9 and 10 day mandibular arch represents a competent dental clone. This single odontogenic clone becomes two separate groups by 11 days, each new clone producing teeth appropriate to its origin. It appears therefore that clones of competent cells do exist within the developing jaw and are capable of producing the different classes of the dentition without extrinsic controls.

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I. Introduction

A. Early Embryology

Fertilization and Cleavage

All higher animals start their life as a single cell-a fertilized ovum. Fertilization increases the respiratory and synthetic activity of the ovum and cleavage is initiated. Through a series of rapid and repeated divisions without increase in size, the fertilized ovum is transformed to a ball of smaller cells approximately 64 in number. This structure is the morula. Cleavage increases the number of cells, providing the basis for cell movement and communication leading to cellular differentiation.

Blastula

Transition from the morula to the next stage, the blastula,involves two major changes. A fluid-filled cavity, the blastocoele,develops in the centre of the morula . Secondly the cells rearrange to form two cell populations. A thin outer layer of cells surrounds the blastocoele while maintaining contact with the outside. These cells will become trophoblast, forming the placenta to establish a nutrient supply. A small compact mass of cells protrudes into the blastocoele. These cells are the inner cell mass and have no external contact (Tarkowski and Wroblewska, 1967). They will form the embryo and its membranes. At the 64-cell stage, the inner cell mass consists of about 15 cells. Of these only about 3 cells will give rise to the body of the embryo (McLaren 1976). Polarity of a mammalian embryo becomes apparent by the late blastula stage, as the embryo approaches gastrulation.

Gastrulation

Gastrulation is characterised by a well ordered rearrangement of cells relative to each other, converting the blastula to a three-layered embryo. These morphogenetic movements

bring together cells that previously had no chance to make contact thereby allowing the opportunity for inductive interactions.

Three primary germ cell layers, **endoderm**, **mesoderm** and **ectoderm**, appear as the result of the migratory cell movements of gastrulation. The first cells to segregate out from the inner cell mass compose the hypoblast. This cell layer forms the extraembryonic endoderm. The remainder of the inner cell mass is the epiblast. The hypoblast and epiblast together form the embryonic disc. A longitudinal thickening appears on the embryonic disc-the primitive streak. Cells of the epiblast migrate through the primitive streak then spread out laterally and anteriorly between the hypoblast and epiblast. In this way the mesoderm , or middle layer is formed. Mesodermal cells first appear in mouse embryos at about 7 days post conception (Theiler 1972).

Associated with the cell movements that form the mesoderm, a cylindrical mass of cells, the notochord, differentiates in the midline just beneath the surface ectoderm. The notochord is an initiating factor in the first major inductive event of the late gastrula, the formation of the neural primordium from the ectoderm overlying the notochord. The neural plate appears first as a thickened plate of ectoderm along the longitudinal axis of the embryo. On either side folds of ectodermal tissue rise up eventually to meet and fuse in the midline, forming the neural tube. A unique population of cells, the neural crest, emerges from the surface ectoderm prior to or during the fusion of the neural folds (fig. 1). A discussion of the neural crest is included in Section B.

Branchial arches

By 7 1/2 days the neural plate is apparent in embryo mice as a shallow groove in the midline. In front of the neural plate is a small buccopharyngeal membrane consisting of ectoderm and endoderm fused together. As mesenchyme flows into the head processes during gastrulation, none intervenes between the layers of the buccopharyngeal membrane. This area of the embryo is the future mouth or stomodeum (fig. 2).

A progressive rising of the neural folds appears to deepen the neural groove by eight days. The head folds begin to bulge as does the tissue all around the buccopharyngeal membrane, giving to the stomodeum the appearance of a deepening pit. The buccopharyngeal membrane remains intact. At this time a marked dorsal flexure of the embryo persists (fig. 3).

The mouse embryo turns and rotates at 8 1/2 days such that the dorsal flexure is reversed(Theiler 1972). At this time two pairs of branchial arches appear as bulges in the mesoderm along the lateral wall of the pharynx. The first of these is the mandibular arch which soon develops a maxillary projection on its cranial side. The second, smaller arch is the hyoid arch. The third and fourth arches are not yet visible. These mesenchymal bulges, the branchial arches, continue to push ventrally around the pharynx between the buccopharyngeal membrane and the developing heart. The two mandibular arches meet and merge in the midline to form the embryonic lower jaw (fig. 12). The maxillary processes, in co-operation with the fronto-nasal processes form the upper jaw. As these growth processes continue to bulge out around the stomodeum, the buccopharyngeal membrane breaks down (about 9 days, 16 somites) (Theiler 1972).

During their early development, 9-11 days in the mouse, the branchial arches are composed of masses of morphologically similar ectomesenchymal cells with an outer covering of ectoderm, about 2-3 cells thick, and a similarly thin endodermal covering (plates 1,2,3,4,5).

Since the mesenchymal cells of the mandibular arch are neural crest derived they are designated **ectomesenchyme** to indicate their origin from ectoderm and to distinguish these cells from mesenchyme of mesodermal origin which is known as **mesenchyme**.

As growth progresses each branchial arch develops a cartilage component and has an associated nerve and blood vessel. The neural crest of the mandibular arch (and its maxillary projection) contributes cartilage, bone and teeth to the jaws.

Development of Teeth

The first histologically apparent sign of differentiation in the mouse mandibular arch is a thickening 4-5 cells deep of the oral ectoderm. From this primary epithelial band, apparent by 11 days, develop the vestibular lamina and the dental lamina. The vestibular lamina appears on the labial side of the dental lamina and eventually forms the labial vestibule. The dental lamina appears first in the incisor area in mice (Hay, 1961). Soon afterward the molar area eptihelium thickens as well (Cohn, 1957). It gives rise to the enamel organs, including ameloblasts, of the developing teeth. Associated with the dental lamina is an accumulation of cells in the underlying mesenchyme which will develop into the dental papilla, giving rise to the mesenchymal components of the teeth; the odontoblasts and the dentin they produce, the cells of the dental pulp and the cells of the periodontium as well (Ten Cate 1969, Ten Cate and Mills 1972). Special stains that indicate high RNA activity demonstrate the presence of these ectomesenchymal cells at 11 days prior to obvious differentiation of the dental lamina (Milaire 1959). The further development of teeth can be described in stages (fig. 4):

1. Bud stage: A dental lamina of 4-5 cells thickness is associated with an accumulation of ectomesenchymal cells.

2. Cap stage: The deeper surface of the epithelial component (enamel organ) invaginates to form a cap shaped structure. The mesenchymal component now consists of dental papilla and dental follicle.

3. Bell stage: Continued growth of the enamel organ converts it to a bell shaped structure with four cell layers, inner enamel epithelium, stratum intermedium, stellate reticulum, and outer enamel epithelium. Hard tissues are laid down in the late bell stage.

4. Root development: As the crown is completed, the cervical loop of the enamel organ progresses apically to form the root or roots.

B. Neural Crest

Cells of the neural crest are known to play a major role in the development of orofacial structures. As previously described the neural crest is formed following the induction of the neural plate and neural folds which rise up to form the neural tube (fig. 1). Cells from the margins of the folds proliferate and migrate into the surrounding mesenchyme passing through the intercellular matrix, moving past other cells and tissues to their final destination (Weston 1970, Johnston and Listgarten 1972). Like gastrulation, this movement of a cell mass in relation to other cell groups allows the opportunity for intercellular communication and possible inductive stimuli. Cells derived from the neural crest population participate in the formation of parts of the nervous system lying close to the neural tube as well as migrating to remote areas of the body where they directly or indirectly, through tissue interactions, give rise to a variety of structures.

The branchial arches are formed by masses of neural crest cells pushing between the endoderm and ectoderm of the pharyngeal wall to create the characteristic series of bulges. The grooves between each set of bulges represent areas where no ectomesenchyme intervenes between ectoderm and endoderm.

Neural crest contribution to skeletal structures

Streams of these cells migrating from the cranial area invade the developing mandibular arch of embryo mice at 8 1/2 days (Theiler 1972). In this location ectomesenchymal cells make a major contribution to the orofacial skeletal structures, cartilage and bone. J. B. Platt, as early as 1893 believed that ectomesenchyme gave rise to the cartilage of visceral arches (Platt 1893). Landacre studied the migration of neural crest cells of *Ambystoma jeffersonianum* by observation of subtle differences in these cells: cell size, staining capacity, pigment and yolk granules (Landacre 1926). He followed the migration of ectomesenchymal cells into the visceral arches where they give rise to cartilage. A number of other investigators confirmed these findings in other amphibian species (Raven 1931, de Beer 1947). Harrison (1918) utilized the

different growth rates of two amphibians *Ambystoma punctatum* and *A. tigrinum* to demonstrate the origin of the branchial arch skeleton from the neural crest. Neural crest of one species when grafted to the other produced a visceral skeleton the size of which was characteristic of the donor species.

Hammond and Yntema (1964) performed deletion experiments on chick embryos to show that removal of the pre-otic neural crest resulted in a deficiency of Meckel's cartilage, indicating the significance of neural crest in avian branchial arch cartilage development.

Isotopic nuclear markers have been used in several experiments to determine the fate of neural crest cells. Weston developed a technique to label embryos with tritiated thymidine, then transfer the labelled neural crest to unlabelled embryos (Weston 1963). The neural crest cells with their radioactive marker can be followed in autoradiographs as they migrate to their final destination. Using this technique Johnston demonstrated that the cartilage and connective tissue of the head and branchial arches of chick embryos are neural crest derivatives (Johnston 1966, 1972).

Le Douarin and her colleagues studied neural crest migration and differentiation using a different cellular marker, one that does not require an isotopic marker (Le Douarin 1969, Le Lièvre 1974, Le Douarin and Le Lièvre 1975). Transplants are instead performed between two closely related species which are compatible, but the cells of which are identifiably different histologically. Embryos of Japanese quail (*Coturnix coturnix japonica*) the cells of which show a large accumulation of heterochromatin, are used in exchange experiments with chick embryos (*Gallus domesticus*), the cells of which have evenly dispersed chromatin. Using this technique Le Douarin and Le Lièvre have confirmed the observation that craniofacial and visceral skeletal elements and connective tissue are of neural crest origin (Le Douarin 1974, Le Lièvre 1974, Le Douarin and Le Lièvre 1975). Johnston's and Le Douarin's *in vivo* techniques attempt to examine the neural crest within its "usual" environment during "normal" development.

To determine the inherent ability of an embryonic tissue to differentiate to mature tissue it is removed from its usual environmental influences and cultured *in vitro*. To some

extent the environment can be controlled to evaluate specific influences on embryonic development. These techniques have been applied to neural crest development to study its capacity to differentiate into cartilage. Drews et al (1972) cultured cranial neural crest of *Triturus alpestris* alone and found that it did not chondrify. When cultured in the presence of developing pharyngeal endoderm 60% of the cranial neural crest cells became chondroblastic. Epperlein (1974) and Epperlein and Lehman (1975) showed that contact with pharyngeal endoderm was necessary for cartilage formation in *Plurodeles waltlii* and that neural crest cells do not preferentially migrate toward the endoderm. Contact with dorsal mesoderm as well as with pharyngeal endoderm was found to bring about chondrogenesis. Corsin (1975) also tested the effects of hyaluronic acid and testicular hyaluronidase on isolated neural crest to determine whether these extracellular matrix products provided the appropriate cue for chondrogenesis. Neither was successful.

The formation of cartilage from neural crest does not depend entirely on contact with pharyngeal endoderm. Neural crest responding to this cue must be of cranial origin. Trunk level crest never forms cartilage or any other skeletal element, indicating an early restriction in developmental capability along the cranial-caudal axis of the neural crest (Raven 1931, 1936). Raven demonstrated in *Ambystoma* that no cartilage formed in the visceral arches when trunk level crest was used to replace the cranial crest. Horstadius (1950) experimenting with *Ambystoma* defined eight regions of the neural crest, each with different developmental capabilities (fig. 5). Segments 1-7 are cranial neural crest. Of these the most anterior, 1 and 2, are not able to form cartilage. Region 4 supplies the cells that form the mandibular arch skeleton. The hyoid and branchial skeletons are formed by regions 5-7. Region 8 is trunk neural crest and cannot form skeletal tissue.

It is evident that some aspects of differentiation are already present in the neural crest at its formation. These experiments also indicate that neural crest cells which are the parent cell mass of the mandibular arch ectomesenchymal population originate from a particular area of the cranial crest and are therefore, under the usual circumstances, capable of skeletogenesis.

Hall (1978, 1980) has used *in vitro* recombination techniques to determine the age and conditions under which avian ectomesenchyme is able or obliged to form skeletal tissue. *In vitro* culture studies allow the possibility of heterochronal and/or heterotopic tissue recombination that give an indication of the interaction which ectomesenchyme requires to become chondroblastic or osteoblastic.

Skeletogenesis of the avian mandible has been extensively studied by Hall. Initially the mandibular skeletal element consists of Meckel's cartilage. As the mandibular arch develops this cartilage regresses and the support of the mandible is provided entirely by bone. Tyler and Hall (1977) have found that the formation of bone must be preceded by interaction between ectomesenchymal cells and mandibular epithelium. Heterochronal tissue recombinations have allowed Tyler and Hall (1977) to determine the embryonic age at which this interaction must occur for the epithelium to provide the correct stimulus to a ready and willing ectomesenchyme. Further experiments with a heterotopic ectodermal component showed that the location of the epithelium is not so important as its mitotic activity (Hall 1980). The mandibular ectomesenchyme gives a specific response, bone formation, to a general stimulus provided by an actively dividing epithelium. A similar epithelial-mesenchymal interaction may be responsible for bone formation in mammals.

Neural Crest Contribution to Teeth

In addition to cartilage and bone the mandibular arch ectomesenchyme of amphibians and mammals is also responsible for tooth formation. Odontoblasts, fibroblasts, cells of the pulp and periodontal membrane producing all of the components of the teeth (except enamel) and their supporting structures, are of neural crest origin. In some cases such as the pharyngeal teeth of fish, ameloblasts may be of endodermal origin, but in most animals they are probably of ectodermal origin. Platt (1867) thought that the dental papilla, including odontoblasts, of *Necturus* were ectomesenchymal tissue. Adams (1924) amd Raven (1931) removed the anterior parts of the neural folds of *Ambystoma* including the neural crest cells. They found that the

adult animal had a reduced number of teeth. De Beer (1947) observed that the cytoplasm of odontoblasts from *Ambystoma* contained black pigment granules indicating their origin from pigmented neural crest. Sellman (1949) used extirpation experiments to localize the area of tooth forming crest in *Ambystoma* to the parts of the neural folds he designated zones 3 and 4, the same location that Horstadius (1950) described as the origin of the mandibular skeletal elements. Chibon (1967) used tritiated thymidine labelled neural crest cells to map migration pathways of amphibian neural crest. He found that cells of the dental papilla originate from the neural crest in a region 30 to 100 degrees from the cephalic neural folds, confirming Sellman's earlier observation (fig. 6).

There is little direct experimental evidence for the role of neural crest in mammalian, as opposed to non-mammalian odontogenesis. Problems encountered in obtaining and growing mammalian embryonic tissue in culture make the acquisition of such data difficult. Gaunt and Miles (1967) suggest that mammalian odontogenesis exhibits the same embryological phenomena as amphibian odontogenesis. In support of this suggestion, Johnston and Listgarten (1972) have shown on tritiated thymidine labelled rat embryos that the pathways of neural crest migration and neural crest contributions to facial development are similar to those in amphibians.

The events leading to the formation of specialized hard tissue in the jaw can be summarized in sequence.

1. Induction of a specialized cell mass, the neural crest, which varies in developmental capability along its cranial-caudal axis.
2. Movement of a specific portion of this cell group to the mandibular and maxillary arches, allowing the opportunity for inductive interactions with other cells, tissues or the intercellular matrix.
3. Localization of neural crest cells in the mandibular and maxillary arches. Here the cells, which to this point are morphologically and histologically indistinguishable from each other, achieve specialization as odontoblasts, osteoblasts, chondroblasts and fibroblasts. In mice

visible differentiation is not apparent before twelve days. Unknown factors arrange these cells, or induce their differentiation, in precise positions forming bones and teeth of the correct size, shape and number in a pattern specific for each species.

C. Pattern Formation

The problem of pattern formation is an intriguing one. All mammals are composed of basically the same tissue types, yet each species has its own pattern of arrangement producing the characteristic morphology. Individual members of a species are always constructed from the same blueprint with only minor deviations. The fore and hind limbs of a particular individual are composed of exactly the same tissues, the cells of which have the same genetic components, however pattern control dictates that each limb is morphologically correct for its location.

Obviously this pattern is genetically controlled but the mechanism by which it is controlled is unknown.

Morphogenetic Fields

A number of theories have been put forward in an attempt to explain the establishment of patterns. One of the earliest of these was presented by Huxley and de Beer (1934) as the theory of "morphogenetic fields". According to this theory:

> "The determination and localisation of organ rudiments is revealed sooner or later by the presence of chemodifferentiated material or morphogenetic substances on certain places which constitute what may be called 'fields' or areas of differentiation of organs. Within the fields the presumptive rudiments become differentiated by progressive chemodifferentiation."

This field concept implies the existence of some external source producing a chemical which then diffuses through the field influencing the cells to differentiate in the appropriate pattern. Neither the chemodifferentiator nor its source have yet been found.

Butler (1939) applied the field concept of pattern formation to mammalian tooth pattern. He noted that teeth are metameric units (members of a graded series) and suggested this was an indication of the action of a morphogenetic field. Butler postulated that tooth rudiments exist in the embryo jaw where they are subject to a graded concentration of an undefined morphogen causing each rudiment to develop into a tooth appropriate for its location. Butler further modified the field theory suggesting that there is an antero-posterior gradient, since there is no tendency to repeat anterior teeth at the posterior end. He also divided his hypothetical field into 3 regions; incisivation, caninisation and molarisation, to account for the three classes of teeth which he defined on the basis of their shape (fig. 7).

Butler went on to describe fossil evidence to show that these fields were subject to evolutionary change - hence the species variation in number and relative size of members of the three tooth classes. According to the field theory the size and shape of teeth, perhaps the number and location as well, are controlled by factors external to the actual primordia. No attempt is made to explain the mechanism of induction of odontogenic competence or the initiation of primordia in a specific pattern along the jaw. Field theory states that all primordia "pre-exist" in the developing jaw and that they are all identical until acted upon by the morphogen. Although Butler's proposals describe what is seen in the mature animal, they do not attempt to suggest a mechanism of action for the postulated morphogen or provide any evidence for its existence.

Positional Information

There is another theory of pattern formation that is similar to the morphogenetic field concept in that an external control influences the cells in specific areas to differentiate appropriately. This is the positional information theory devised by Wolpert (1978). He defines pattern formation as the spatial organization of cytodifferentiation i.e. in any area of undifferentiated cells, if each cell "knows" which cell type it is to be then the morphologically correct structure will be formed. Wolpert goes on to suggest a positional information theory

whereby each cell receives information about its position which it uses to differentiate appropriately. He uses the tri-color French flag as an analogy for development. In a rectangular array of undetermined cells each has the ability to be white, blue or red. They form the appropriate arrangement of red, white and blue bands on the basis of information received from the boundaries regarding their position in the rectangle. In general terms this would mean that cells in any area of a developing embryo receive a signal regarding their position and differentiate according to their genetic capability. Presumably the cells have a number of developmental options, from which they select on the basis of the positional information they receive. Specifically, the cells of the mandibular arch become odontoblasts, osteoblasts, fibroblasts or chondroblasts depending on their location. The cells must not only receive and acknowledge the signal, they must remember it as well since mandibular arch tissue will still develop teeth, bone, cartilage and periodontal membrane in a normal relationship when grown in culture away from any positional information source (Glasstone 1936, Kollar and Baird 1968, Gerber et al 1974, Lumsden 1979). Where a positional signal emanates from in normal development, or its nature, is not clear.

One possibility by which cells could determine their position in a growing system has been suggested by Wolpert, Lewis and Summerbell (1975). They use the chick wing limb bud as a model. As the bud grows out as an extension from the body its tip is a specialized region they have named the "progress zone". Cells in this zone divide constantly, but the width of the zone remains constant so cells are left behind as the limb grows out further from the body. Cells closest to the body have left the progress zone first, those at the tip have spent the most time in the progress zone. This information is used by the cells as positional information in their differentiation. Although cells may appear to "behave according to their position" (Wolpert 1978), we should not assume that they somehow "read" their relative position from some external source. This again is an attempt to explain a mechanism of pattern formation that really only describes what is seen in the fully formed individual.

Other External Controls

Some researchers have suggested that the patterns seen in later developed structures are imposed secondarily by other, previously present, tissues. This idea has been applied to the patterned initiation of tooth primordia in the developing jaw. Both blood vessels and nerves have been implicated.

Gaunt (1959) described capillary networks in the developing jaws of cat embryos, indicating that these capillary networks were visible prior to overt differentiation of tooth primordia and therefore a possible cause of odontogenic differentiation in specific areas. Dalcq (1953) in a similar study used a special stain to indicate metabolically very active cells by their high RNA concentration. He postulated the reverse of Gaunt's suggestion, stating that the metabolically active cells (possibly pre-odontogenic) attracted the blood vessels. In this case the blood vessels arrive secondarily rather than causing the differentiation of cells.

Slavkin (1974) suggested that ectomesenchyme migrates into the developing mandibular arch via the pathways of the sensory fibres of the trigeminal nerve. As the neural crest cells migrate along this nerve pathway they accumulate in areas appropriate for odontogenesis. This proposal implies that some migrating crest cells are pre-committed to become odontogenic. They need only to accumulate in the correct place to form a tooth primordium. No experimental evidence has been presented to support this hypothesis.

Others have suggested a possible inductive relationship in which the nerve fibres would in some unknown way cause the already present ectomesenchymal cells to become odontogenic. Pearson (1977) noted a relationship between fine nerve fibres and areas thought to be preodontogenic in human embryos. At best this evidence is circumstantial.

Kollar and Lumsden (1979) have demonstrated the presence of fine nerve fibres in areas presumed to be pre-odontogenic in mice. They support their suggestion of an inductive relationship by pointing out the known relationship between the ingrowth of nerve fibres and the development of sensory organs associated with those nerves, such as vibrissae (Van Exan and Hardy 1980) and taste buds (Farbman 1972). No direct evidence is available to support this

theory in relation to tooth induction. Later research by Lumsden tends to disprove his earlier proposal (Lumsden 1984). He demonstrated that teeth will form in culture from very early mandibular arch, at a time when the mandibular nerve has not yet grown into the area.

Clones: Intrinsic Control of Pattern

There is a theory of pattern formation which does not require the presence of extrinsic controls such as morphogens, positional indicators, nerves or blood vessels. Osborn outlines this theory in "Morphogenetic Gradients: Fields vs. Clones" (Osborn 1978a). He believes that "patterns are self -generated rather than 'controlled' from the outside". He uses the example of a tooth class to further explain the clonal theory of shape generation. According to Osborn a clone of dentally competent cells arises in the mandibular arch. The cells of this group are in the same differentiation compartment and are descendants of the neural crest cells described previously. It is not clear how the clone arises. An inductive event may stimulate the cells of a certain area (or areas) to become odontogenic, or cells previously determined to become odontogenic may accumulate in the appropriate area. By whatever means the clone originates, its cells continue to divide until competence to initiate a tooth primordium is achieved. This primordium is the stem progenitor. The clone of cells continues to expand away from the first primordium (the clone may grow anteriorly and posteriorly and inferiorly - to produce the secondary dentition). Eventually the clone again reaches a size at which another primordium can be initiated. As the mandibular arch grows space becomes available for the expanding clone to produce more primordia. Later initiated primordia are derived from cells that have divided more times than those of earlier primordia. "There is a gradient in cell ancestry of successive primordia" (Osborn 1978a). Osborn feels that this gradient of cell ancestry is reflected in the gradient of tooth size and shape (fig. 8).

The clone model requires only that a dental clone be initiated and continue to grow to produce the appropriate dentition. No extrinsic influences are required after the clone is established. Unlike the field model, the clone model can be tested by isolating the clone and

allowing it to develop in culture. The clone should be able to produce its entire tooth class without the aid of extrinsic factors. Lumsden (1978) has demonstrated the development in culture of M1, M2 and M3 the complete molar class along with their supporting tissues, from the first molar primordium (the stem progenitor) of a 13 day mouse embryo.

The 13 day embryonic tissue cultured by Lumsden represents the dental clone developed to a point when the first primordium is initiated. As predicted by the clone model, this primordium-clone continues to develop without extrinsic factors, producing 2 more primordia of the appropriate size, shape and relationship to each other (consideration being given to the restricted physical environment of the eye used for *in vivo* culture).

Hypothetically one could use tissue from an earlier stage to produce the same molar series. The early odontogenic clone, however, cannot be distinguished from the surrounding cells. Cultures of early mandibular arch invariably will include surrounding cells which are not actually part of the clone but also give rise to ectomesenchymal tissues such as bone, cartilage or fibrous tissue (Lumsden 1978).

The developmental history of the cells comprising the dental clone and the circumstances that delineate the clone as a discrete unit are largely speculative. Osborn has offered a sequence of development from neural crest via a series of step-wise determinations to the dental clone, as well as the cell groups that form the other ectomesenchymal orofacial components (Osborn 1978b). This theory will be more fully discussed in the following section.

D. The Unit of the Dentition

A consideration of the development of teeth must address the question of the unit from which the teeth develop. What is the smallest, earliest identifiable group of cells that could be considered truly odontogenic. ie. capable of producing the entire dentition or subdivisions of the dentition? Individual tooth primordia obviously give rise to teeth however, as Butler (1939) noted, "teeth must be considered as metameric organs evolving as part of a system and not as individual units". Although Butler used this idea to launch his morphogenetic field concept, it

also suggests that the dentition is represented developmentally, genetically, as a whole rather than as individual teeth.

Classes of Teeth

Mammals typically have a dentition that represents some variation of the general pattern: incisor, canine, molar. Different species have a variable number of teeth representing each class. Traditionally these classes have been defined by position. The maxillary canine is defined as the tooth directly behind the premaxillary/maxillary suture; hence there can be only one canine per quadrant. The mandibular canine is the tooth that occludes anterior to the maxillary canine. The incisor class is anterior to the canine while the molar class is posterior to it. When more than one member is present in the incisor or molar class a shape/size gradient is apparent. Butler used this classification system to develop the field concept of tooth development. He felt that the presence of three different classes of teeth necessitated the existence of three morphogenetic fields to produce the appropriate shape gradients (fig. 7). This maxillary suture system of classification, which is firmly entrenched in the zoological literature, is often used to homologize the tooth classes of the different species, an assumption which may lead to erroneous conclusions since the classification is not based on the developmental history of the teeth.

Osborn has redefined the concept of tooth classes based on his clone model of tooth development (Osborn 1978a). As previously described, the dental clone produces a series of tooth primordia the shapes of which are graded according to the time at which they are budded off the growing clone. The shape/size potential of a clone may fall steadily (increasingly smaller teeth) or may rise then fall (small increasing to large then smaller again) but never rise, fall, then rise again. The result of this scheme would be a graded series of teeth such as the molar or incisor class.

The tooth classes recognized by this system are not entirely synonymous with those defined by the maxillary suture and field theory system. Osborn (1978a) cites the example of

Artiodactyls in which the lower canine resembles an incisor. The field theory requires that the incisivation field "spill over" into the canine area to generate this particular configuration. However the clone model classifies this tooth as an incisor since its size and shape compared to the adjacent teeth indicate that it is derived from the incisor clone.

The Dental Clone as the Odontogenic Unit

To produce the typical mammalian dentition three odontogenic clones are required; anterior, middle and posterior (fig. 8). In a eutherian mammal the anterior clone grows posteriorly and inferiorly producing the deciduous incisors and their permanent successors. The middle clone usually generates only the deciduous and permanent canines. The posterior clone must grow anteriorly and inferiorly to produce the deciduous molars and the premolars, while the permanent molars arise from the posterior growth of the same clone. Osborn also speculates that each dental clone gives rise to the tissues associated with their respective tooth classes. ie. alveolar bone and periodontal membrane. Lumsden (1978) supported this claim by demonstrating the development of 3 molars plus alveolar bone and periodontal membrane from the initial primordium present in a 13 day mouse embryo. The origin of alveolar bone from the odontogenic clone may explain its difference from basal bone despite their morphologic and histologic similarity.

Since clone development cannot be directly observed we must rely on tissue culture to provide evidence in support of the clone theory. Kollar and Baird (1968) have divided the 14 day embryonic mouse mandible into three sections corresponding to anterior, middle and posterior segments. The anterior segment cultured *in vivo*, in the anterior eye chamber of an homologous adult mouse, produced the single incisor which comprises the complete mouse incisor class. The middle segment, diastema region, produced bone, cartilage and fibrous tissue but no teeth. Mice have no canine class of teeth presumably because no dental clone develops in this area. Kollar and Baird (1969, 1970a,b,c.) performed a number of recombination experiments to demonstrate the deficiency of odontogenic capability in the diastema

mesenchymal component. Kollar and Baird's grafts of posterior mandibular segments produced molar teeth plus some bone and periodontal membrane, but they do not mention the formation of more than one tooth. The posterior segment of embryonic mandibular arch includes the posterior clone and forms the entire posterior dentition when cultured long enough (Lumsden 1979). Glasstone (1967) had earlier described the formation of two teeth from the posterior mandibular arch segment, but attached no significance to it.

The early existence of a clone, prior to dental lamina formation, is not directly observable since the cells comprising the clone are not visibly different than the surrounding ectomesenchymal cells. The presence of the clone can be inferred from the ability of early mandibular arch tissue to produce teeth in an isolated environment either *in vivo* or *in vitro* . Several attempts have been made to culture mandibular arch tissue prior to 12 days when the first molar and incisor primordia are already present as a dental lamina and associated accumulation of ectomesenchynal cells.

Dryburgh (1967) found that *in vitro* organ cultures of whole 8 day mouse mandibular arch failed to produce teeth. The mandibular arch of an 8 day post conception mouse embryo is just beginning to form as a barely visible bulge (Theiler 1972). The extreme difficulty in removing such a structure for culture suggests that Dryburgh may have numbered the days starting with day 1 as the morning following mating rather than day 0. No mention is made of the method of determining embryo age in Dryburgh's work (Dryburgh 1967). Starting with day 1 would mean the day 8 embryo would have been day 9 by our method of determining age. Dryburgh, in the same study, found that cultures of 9 day (10 day?) mouse mandibular arch undergo histodifferentiation, and that 10 day (11 day?) mandibles demonstrate morphodifferentiation when the entire arch is cultured.

Gerber et al (1974) observed the development of teeth (molar and/or incisor) in 27 of 55 intratesticular grafts of whole 9 day mouse mandibular arch.

Lumsden (1978) cultured posterior segments of 12 and 13 day mouse mandibular arch *in vivo* . He found one or more molar teeth forming in the majority of these grafts. Lumsden

(1984) later cultured segments of 10.5 day mandibular arch using the same intraocular technique. He found well formed incisor teeth in cultures of anterior segments from these early embryos. The focus of incisor-forming tissue was present between 15 to 30 degrees from the midline. Molar forming tissue was found to centre in the cells 45 to 60 degrees from the middle. The 10.5 day mouse mandibular arch demonstrates two separate areas of odontogenic tissue with only a small area of intervening nonodontogenic tissue (fig. 9).

The origin of these clones remains obscure, as does the loss of the middle clone. As described previously, the component cells initially came from the neural crest. It is possible, but unlikely, that within the premigratory crest is a subpopulation of a few cells determined to be odontogenic. This would necessitate the precise migration of these cells via a specific pathway to exactly the appropriate location for tooth formation. Neural crest cells appear to follow a more opportunistic pathway (Weston 1970, Johnston and Listgarten 1972, Le Douarin 1982). They enter a migration pathway appropriate to their location and orientation, and within inherent limits develop into mature tissues according to environmental influence (Weston 1970). In culture premigratory neural crest cells develop few specialized cells, other than melanoblasts, depending on culture conditions (Greenberg and Schrier 1977). It would appear that the neural crest cellls, while they have the ability to become odontogenic, require the interactions present in a normally developing embryo to form a dental clone.

Osborn (1978b) has proposed a sequence of differentiations, beginning with cranial neural crest, that could produce the cell groups required to form the four basic clones responsible for the formation of all orofacial ectomesenchynal structures in a generalized mammal (fig. 10). Three of the clones are the odontogenic clones previously described. They form the teeth and all supporting structures. The fourth clone forms the basal bone.

Osborn's theory borrows the concept of a clade from evolutionary theory. A clade includes all the members of a taxon or in this case the members of a cell population. All members are alike. The clade can be directly ancestral to only two other groups. In terms of cell differentiation this would mean that no cell or cell group is multipotent. A cell (or clone) may

chose only one of two differentiation options. Through a series of these bipotent differentiations one cell (or clone) can give rise to several cell types.

The neural crest can, through sequential bipotency, give rise to all cell types required to form the teeth and jaws.

The developing mouse mandibular arch is at variance with the generalized mammal in one aspect. The canine class of teeth is not represented in mice. Apparently the clone responsible for its formation does not develop. There are a number of possible explanations. According to Osborn's cladistic scheme the canine clone (C.C.M.) is derived with the molar clone mesenchyme (M.C.M.) from a common cell mass, the posterior clone mesenchyne (P.C.M.). If the P.C.M. does not develop to a large enough mass to form two groups, only one would exist, which apparently forms the molars. Alternatively the stimulus which causes differentiation of the P.C.M. to M.C.M. and C.C.M. may be missing, or if present the stimulus is not recognized.

The presence of these clones cannot be demonstrated histologically as there are no visible differences between ectomesenchymal cells making up the embryonic mandibular arch. For this reason the presence of a clone must be inferred from the ability of mandibular arch tissues to produce teeth when allowed to develop in isolation from the normal environment. Cultured anterior, middle and posterior segments of early mandibular arch should contain the odontogenic clones and should produce teeth of the appropriate shape and number. The anterior segment, containing the I.C.M. should produce an incisor. The middle segment, if there is no C.C.M. would be expected to produce no teeth, while the posterior segment containing the P.C.M. or M.C.M. would produce the entire mouse molar class, M1, M2, and M3.

The present investigation attempts to trace the development of dental clones from the ninth day of embryogenesis, the time when the mandibular arch first becomes apparent in mouse embryos. By cutting the developing arch into 3 segments, followed by culture in the anterior eye chamber, it was hoped to be able to identify the appearance and subsequent fate,

during days 9, 10 and 11, of dentally competent cells.

II. Materials and Methods

The rodents selected for use as donors and recipients in this study were Balb-cCR mice. These inbred albino mice are small, cheap and easy to keep. They also respond well to intraocular grafting and as an inbred strain they are immunologically tolerant. Much previous work has been done using these or similar strains of mice in the study of tooth development (Kollar and Baird 1969, 1970a,b,c., Lumsden 1979). The Balb-cCR mice were obtained from University of Alberta Animal Services, and kept under standard conditions. Their gestation period is 20 days.

A. Donor Material

In mice the estrus cycle varies from 4 to 5 days (Bronson et al 1966). Female mice living in a colony under the same conditions tend to have synchronous estrus cycles. Mating occurs at night during the estrus phase. Vaginal smears taken to reveal the stage of the estrus cycle were used to determine the best night for mating. The estrus stage as revealed by vaginal smear was found to be a more reliable indicator of mating than the capricious vaginal plug.

Two young females (8-12 weeks) believed to be at or near estrus were placed with one young male (8-12 weeks)in a cage overnight and checked for vaginal plugs the following morning. The male was then removed from the cage and the females kept until required for the experiment. The morning following mating was counted as day 0. On the required day (9-11) after mating a pregnant mouse was killed by cervical dislocation and her uterus, with embryos, removed to a dish of sterile Hanks' Balanced Salt Solution (H.B.S.S.). The uterus was rinsed then placed in a second dish of sterile H.B.S.S. in a laminar flow hood. All subsequent grafting procedures were carried out in a laminar flow hood using sterile technique. Embryos were removed from the uterus with their membranes intact and transferred for dissection to H.B.S.S. with 10% fetal calf serum (F.C.S.) in a 3-well glass slide. The bottoms of the wells in the glass slide were covered with black wax to prevent damage to the dissecting needles and to provide a contrast against which the translucent embryo was visible. All dissection was done

under H.B.S.S. with 10% f.c.s. The external features of embryos were compared to stages described by Theiler in "The House Mouse" (1972) as a cross check on the accuracy of developmental age.

At this early stage of development the embryo is smaller than the surrounding membranes. Careful removal of the thick membranes with ultrafine forceps was necessary to avoid damaging the embryo. Once the embryo was detached from its membranes the head end was removed using two fine dissecting needles in a scissor-like motion. The head was then placed in a clean well of H.B.S.S. with 10%f.c.s. before removal of the mandibular arch with dissecting needles.

The mandibular arch, once obtained, was cut into segments beginning from the posterior end of one side and working inwards. The mandibular arches were not cut down the midline immediately as it was difficult to reorient the two pieces once they were separated i.e. it was difficult to distinguish the cut midline from the posterior end.

The posterior segment was cut from one end using fine tungsten needles. This segment was then drawn up with a pipette and placed in H.B.S.S. with 10% f.c.s. in a labelled well in another 3 welled slide. The middle was similarly removed and placed in the next labelled well. The arch was then turned around and the posterior and middle segments of the other side cut and placed in the labelled wells of a separate slide. Finally the anterior segment was cut through the midline and each anterior segment placed with the appropriate middle and posterior segments. The glass slide containing these segments was then enclosed in a petri dish and set aside at 4 degrees Centigrade for later grafting.

Some mandibular arches were immediately fixed in neutral buffered formalin (N.B.F.) and were sectioned and studied histologically to determine the stage of mandibular arch development prior to grafting. Some whole heads were fixed as well. Segments of mandibular arch were not fixed for use as a control as it was found that these fragile segments were too small to see and handle throughout processing.

B. Anesthesia and Grafting

When dissection and isolation of donor material was complete, the graft recipients were anesthetized. This *in vivo* technique was chosen after numerous trials of *in vitro* techniques failed to produce adequate results. None of the culture media used *in vitro* permitted optimum development of the graft tissue.

The intraocular site chosen in preference to the subcapsular area of the kidney has a number of advantages, not the least of which is accessiblity. The graft tissue once in place under the cornea remained viable in the aqueous humor until vascularity was established (Fleming 1952, Kollar and Baird 1969, Lumsden 1979). If the surgery was carefully and cleanly accomplished the cornea did not scar or become opaque. The graft was therefore visible throughout the culture period.

Young (6-10 weeks) males of the same inbred strain were chosen as graft recipients. Mice of this age tolerate the anesthesia more readily than younger or older mice. At six weeks the eyes are large enough to allow the surgical procedure while the cornea is not as resistant to incision as it is in older mice.

Sodium pentobarbital was used as the anesthetic agent (.2 ml. of 10 mg./ml. solution injected intraperitoneally). Very deep anesthesia is required as the palpebral and corneal reflexes must be inhibited to allow the delicate surgical procedure without the animal blinking. Damage to the cornea, and more especially the iris, was avoided to ensure a successful graft. Unfortunately this depth of anesthesia is perilously close to death. The dose of sodium pentobarbital needed to bring an individual mouse to this level of anesthesia varies somewhat, a factor which inevitably results in the death of some mice from anesthetic overdose, while others of similar weight given the same dose could not be used for surgery as the anesthetic was not deep enough.

Anesthesia is adequate when the mouse fails to respond to a light touch on the cornea. The eye remains bright red and the pupil is very constricted, an added advantage in placing the graft against the iris. If the pupil is slightly dilated and the eye dark red, the mouse is unlikely

to survive the surgery although it may survive the anesthetic if simply left undisturbed.

Once an appropriate level of anesthesia was reached the animal was placed under the dissecting microscope with its right eye directed up. A saline wash was used to eliminate debris from the eye area. Gentle finger pressure was exerted on the eye causing it to bulge outward slightly and making the cornea available for incision. The first incision was made using a sharp single edge ophthalmic blade (Beaver no. 65 disposable mini blade) (fig. 11). By holding the blade at an angle, starting at the outer edge and cutting along a radius toward the centre of the eye, damage to the iris was avoided. An advantage of using albino mice is the lack of iris pigment that makes the iris blood vessels highly visible and therefore avoidable. In this way hemorrhage that could jeopardize the success of the operation was minimized. A second cut was made back toward the periphery of the eye using ophthalmic micro-spring scissors, thereby forming a triangle shaped flap in the cornea .

These incisions allowed the aqueous humor to escape from the eye and it was necessary to remove this fluid with a kimwipe before proceeding with the actual grafting. The aqueous humor reforms rapidly following surgery (Lumsden 1978). The extremely small size and delicacy of the graft material required special techniques to transfer the graft into position under the cornea. Metal instruments such as probes or forceps cannot be used to handle the grafts. A 3-well slide containing anterior, middle and posterior segments was placed under the dissecting microscope and the segment to be grafted picked up in a fine glass tube connected to a short length of rubber tubing and a rubber bulb.

The glass tube was made from a disposable 5 microliter micropipette (Drummond micro-caps) with one end reshaped to a bevelled point. This bevel was found to be necessary as the blunt end of the capillary tube was far too large to enter the small corneal incision. The bevel was created with a rotating fine dental polishing stone followed by ultra-fine polishing discs to give a smooth finish. Due to the fine bore size of the micro-pipette extreme care must be taken when drawing up the graft to avoid sucking it right up into the rubber bulb and losing it. A short length of rubber tubing was added to give finer control of the pressure within the

micro-pipette. A very slight pressure on the tube, or bending, was enough to draw up the graft and expel it. Once the graft was picked up in the micro-pipette, the bevelled end was inserted, bevel up, through the corneal incision. The graft was then expelled into the eye, under the cornea and on top of the iris, and the tube carefully withdrawn. Caution must be taken to avoid drawing the graft back out with the tube. Grafts of 9-day tissue were so small and translucent it was often difficult to tell if the graft was in place or not. Some grafts were undoubtedly lost unknowingly during the procedure. After initial placement the graft was gently pushed around the eye to the side opposite the incision by pressure on the external surface of the cornea. This repositioning was to avoid any possible loss of grafts through the incision. However it was noted that the incision closed very rapidly, even becoming adherent before the anesthetic wore off. Therefore it is unlikely that any grafts were lost through the corneal incision after their initial placement.

The mice with grafts were marked with an ear punch to indicate the type of graft, and placed in appropriately labelled cages. Since death due to hypothermia is a possibility with this type of anesthetic and surgery (Lumsden 1978) the recuperating mice in their cages were placed in an incubator at 26 degrees Centigrade. This temperature was chosen as a compromise between causing death either due to hypothermia or due to the increased toxicity of the anesthetic drug at higher temperatures. The toxicity of nembutal increases with increase in temperature. At 26 degrees Centigrade the toxic dose is 80 micrograms/gram, but falls to 10 micrograms/gram at 36 degrees Centigrade. (Keplinger, Lanvier,and Diechmann 1959) Inevitably, despite precautions, some mice failed to recover from the anesthetic. The toxicity of the drug is also increased by surgical shock. 21% of the mice that received grafts during the experiment suffered anesthetic death. These losses were high for initial experiments but decreased as technical skill improved. Mice that did not apear to be tolerating the anesthesia well were not operated thereby decreasing the loss of mice with grafts.

When the animals had fully recovered from the anesthesia, they were replaced in the animal room and kept under standard conditions for three weeks. After this time they were

killed by cervical dislocation. The grafts were retrieved and immediately fixed in 10% N.B.F. As calcified tissue was present in many of the harvested grafts a decalcifying agent was required following fixation. Grafts were placed in formal citrate (formic acid, sodium citrate) for 1-2 days depending on their size to ensure complete decalcification. They were then dehydrated through ascending grades of alcohol, cleared in xylene and permeated with liquid paraffin. After imbedding in paraffin blocks they were serially sectioned at 5 microns for histologic study. All graft sections were stained with hematoxylin and eosin. Ungrafted mandibular arches and whole heads were processed and sectioned by the same method (decalcification was not necessary for early stages). The sections were stained with either hematoxylin and eosin or Linder's nerve stain (Linder 1978).

Sections of retrieved grafts were examined for the presence of dental structures----dentin, odontoblasts, enamel matrix----in the form of tooth primordia. Wax models were made of some of these tooth primordia to determine their 3-dimensional shape. The presence of bone and/or cartilage was also noted. The outline of bone structure was included in wax tooth primordia models to study the relationship between bone development and tooth development.

III. Results

A. Controls

Sectioned and stained whole heads of 9, 10, and 11 day embryos were studied to determine the developmental stage of mandibular arch tissue prior to grafting. In addition mandibular arches were removed from 10 and 11 day embryos and examined histologically. This was not done with 9 day embryos as the mandibular arches were too small to handle through processing and imbedding. Some 12 and 13 day heads were examined to observe the first visible signs of tooth development.

9 Day Mandibular Arch

The mandibular arch was apparent as a small bulge protruding between the forebrain and the developing heart. The external covering was a layer of simple cuboidal or low columnar epithelium (plate 1). This epithelium was generally one cell layer thick, although two cell layers were present on the oral surface (plate 2a,b). Ectomesenchymal cells formed the bulk of the arch. These irregularly shaped cells were uniformly densely packed throughout the arch. Intercellular spaces evident on sections of tissue fixed in neutral buffered formalin or formal calcium were due in part to contraction of the cells during fixation (Bancroft and Stevens 1982). Mandibular arch ectomesenchymal cells at this stage stained uniformly with hematoxylin and eosin. Their cytoplasm was eosinophilic and appeared homogeneous. Nuclei were prominent and basophilic. Mitotic figures were apparent throughout the arch. Nucleated blood cells, primitive erythroblasts, were present within endothelial walled capillaries. Although the trigeminal ganglion was readily visible at 9 days peripheral nerve fibres were not apparent on the hematoxylin and eosin sections. Neither did sections stained with Linder's nerve stain show any evidence of nerve fibres in the 9 day mandibular arch.

The right and left mandibular arches were joined only by a thin membrane of endoderm and ectoderm passing between the developing heart below and the buccopharyngeal membrane

above (fig 12). A shallow broad groove or trough was apparent in the midline of the mandibular arch where the ectomensenchymal cells had not yet pushed through to merge with the cells of the opposite side.

10 Day Mandibular Arches

The bulge of the mandibular arch was more prominent, with the maxillary projection now apparent on its cranial and dorsal surface. Caudal to the mandibular arch and separated from it by a branchial cleft was the much smaller hyoid arch. The epithelium covering the mandibular arch on its oral surface was cuboidal or low columnar, 1-2 cells thick. The external and pharyngeal surfaces had a thinner covering of simple squamous epithelium (plate 3,4a,b).

The ectomesenchymal cells maintained their irregular outline and were still densely packed. In the central area the cells appeared to be oriented with their long axes in the antero-posterior direction. Their staining characteristics remained uniform. Mitotic figures seemed to be as abundant as they were in 9 day specimens. No differentiation of ectomesenchymal cells was apparent. Hemotoxylin and eosin sections did not reveal nerve fibres within the arch, although branches emerging from the trigeminal ganglion were evident. A few fine nerve fibres were made visible in the posterior area of the arch with Linder's nerve stain. None of these tiny fibres appeared to touch or approach the periphery.

The ectomesenchyme of the right and left sides was not yet confluent. The shallow midline groove was now more narrow as ectomesenchymal cells merge across the midline (fig. 13).

11 Day Mandibular Arch

The mandibular arch was now well developed with the mesenchyme of right and left sides confluent through the midline. A shallow groove still marked the midline. The maxillary projection was merging with the frontonasal process to form the upper jaw. The branchial cleft separating the first and second arches remained as a shallow groove on the external surface (fig

14).

The oral surface epithelium was at least 2 cells thick on an 11 day mandibular arch (plate 5). The basal cells were columnar or cuboidal. Those closer to the surface were cuboidal. The ectomesenchyme was still densely packed and could not be described as a denser accumulation under the oral surface epithelium. There was no apparent differentiation of ectomesenchymal cells at this point when hematoxylin and eosin stain was used (plate 6a). Sections stained with Linder's nerve stain showed the mandibular nerve in the central portion of the arch with branches extending toward but not touching the periphery (plate 6b).

To this point the mandibular arch had changed in size and shape, but had maintained the basic configuration of a mass of cells covered by a thin epithelium. Differentiation of cells was not histologically visible.

12 day Mandibular Arch

Two discrete thickenings of the oral epithelium, 3-4 cuboidal cells deep (Plate 7), were apparent along the antero-posterior axis of the mandibular arch. This was the dental lamina stage of development. One of these thickenings was in the incisor area the other represented the future molar area. Associated with the epithelial thickenings was an accumulation of ectomesenchymal cells which could be distinguished from the surrounding cells by their denser packing (plate 7). The anlage of Meckel's cartilage was now apparent as a central round area of more lightly stained cells separated slightly by intercellular matrix. Bulges in the floor of the pharynx indicated the beginning of tongue development.

13 day Mandibular Arch

The anterior and posterior tooth primordia entered the bud stage of development as the epithelial thickenings invaginated the underlying accumulation of odontogenic mesenchyme. Meckel's cartilage, the mandibular nerve and blood vessels were apparent. Vibrissae follicles were developing in the maxillary area as invaginations of the snout epithelium.

B. Grafts

Histology of 9 Day Grafts

Anterior Segment Grafts

Initially 9 day arches were cut into 3 segments per side; anterior, middle and posterior, and each segment was placed separately as an intraocular graft. As the ectomesenchymal portion of the mandibular arch does not extend across the midline at this stage, the anterior segments consisted almost entirely of epithelium and were extremely small and fragile. Attempts to graft these anterior segments consistently failed. Only one graft, composed almost entirely of a cyst, was retrieved. To enhance the viability of these grafts the rest of the anterior segments were grafted without cutting across the midline. The right and left anterior segments of 19 nine day mandibular arches were grafted as a-a units while the middle and posterior segments were grafted separately. Thirteen of the a-a grafts were retrieved and studied histologically. Tissue types present in some, but not all grafts were epithelium, loose connective tissue, salivary gland acini, muscle and cartilage (plate 8). No bone was found in the anterior segment grafts of this stage. No teeth or tooth primordia were found. Some inflammatory cells were present in all grafts. With few exceptions this inflammatory infiltrate was not a prominent feature. Some grafts, possibly infected or immunologically rejected were densely packed with lymphocytes.

Middle Segment Grafts

Forty-four grafts were placed, of which 29 were successful. These grafts developed tissues similar to those seen in the anterior segment grafts. Epithelium, loose connective tissue, salivary gland acini, muscle and cartilage were present. In addition, bone was found in the middle segment grafts. Hair follicles were present with associated sebaceous glands. Lymphocytes were again seen in grafts with dead or dying tissue.

Teeth were found in 8 of the 28 successful grafts (plate 9a,b). These teeth had developed enamel, dentin and pulp in the typical configuration. Well-mineralized enamel

appeared only as an artifact space on these decalcified sections. In some instances of incomplete mineralization a few shreds of organic enamel matrix were still visible. The tooth crowns were formed in a near normal shape, but there was no root formation. Molars were identified by folding of the enamel surface to form a multicusped shape. Disturbance of cusp formation resulted in altered crown shape in some grafts. All teeth were associated with bone but separated from it by fibrous connective tissue.

Posterior Segment Grafts

Tissues produced by grafts of posterior segments were similar to other grafts of this stage: epithelium, loose connective tissue, cartilage and bone. No teeth were found in posterior segment grafts.

Whole Arches

Five 9 day mandibular arches were divided in the midline and each side placed as a whole graft. One of these was lost during the grafting procedure. One failed while eight were retrieved. Histologic features of the whole grafts were similar to those seen in grafts placed as segments. Five whole arch grafts yielded teeth plus their supporting bone. Two grafts had a single molariform tooth. Of the two grafts that produced two teeth, one had an incisor plus a molar and the other had two molars. Teeth with enamel on only one surface and no folding of the surface were identified as incisors. One graft produced three teeth, an incisor and two molars (plate 10). Those grafts that did not produce teeth showed cysts, bone, cartilage and hair follicles.

Nine Day Grafts

Table I-a

9-Day Mandibular Arch Segment Grafts

Grafts placed	a[1]	a-a[2]	m[3]	p[4]
	5	19	44	44
Grafts retrieved				
with tooth/teeth	0 (0%)	0 (0%)	8 (18.1%)	0 (0%)
without tooth/teeth	1 (20%)	13 (68.4%)	20 (44.4%)	30 (68.1%)
total	1 (20%)	13 (68.4%)	28 (63.6%)	30 (68.1%)
Failed grafts (failure of graft to survive)	3 (60%)	5 (26.3%)	15 (3.4%)	10 (22.7%)
Anesthetic death (failure of host to survive)	1 (20%)	1 (5.2%)	1 (2.2%)	4 (9%)

9 day failure rate (Total) = 40:112 (35%)

1. a represents anterior segments separated as two grafts
2. a-a represents right and left anteior segments joined as one graft.
3. m represents middle segment grafts.
4. p represents posterior segment grafts.

Table I-b

Whole Arch Grafts (Hemi-mandibles)

Total	with teeth	without teeth	failed
9	5	3	1

Table I-c

Number, Shape and Location of teeth Found Developing in 9-Day Grafts

Mouse Number	a	a-a	m	p
9-2 (ii)	—	—	one molar	—
9-2 (iii)	—	—	one molar	—
9-5 (i)	—	—	one molar	—
9-6 (i)	—	—	one molar	—
9-6 (i)	—	—	two molars	—
9-6 (ii)	—	—	one molar	—
9-6 (iii)	—	—	one molar	—
9-9 (iv)	—	—	one molar	—

Histology of 10 Day Grafts

Anterior Segment Grafts

All 32 ten day anterior segments were grafted separately. None was left joined at the midline. Eighteen grafts were retrieved. The histology of these grafts was similar to those from nine day tissue (plate 11). Epithelium, loose connective tissue, glandular tissue, and cartilage were present, but some tissues were present that were not found in grafts of earlier tissue. Bone and hair follicles were now apparent.

One tooth was retrieved from an anterior graft (plate 12). It had an incisiform shape and was enclosed by bone at its apical end. Enamel, dentin and pulp were present in the configuration of a normal tooth. A follicle surrounded the tooth separating it from the bone.

Middle Segment Grafts

Thirty-two grafts were placed, of which 28 were successful. Middle segments produced epithelium, connective tissue, salivary gland acini, muscle, hair follicles with sebaceous glands, cartilage and bone. Fourteen grafts produced teeth all of which were molariform surrounded by a follicle and bone (plate 13). Of these fourteen tooth producing grafts, four had two molars. In 3 cases the two teeth were in the normal side by side relationship of M1 and M2. The other graft had two teeth in an aberrant relationship. In one additional case an M3 was present as well.

Posterior Segment Grafts

Of 32 posterior segment grafts, 20 were retrieved. Tissues produced were epithelium, connective tissue, muscle, glandular tissue, cartilage and bone. No teeth were found in any posterior segments.

Whole Arch Grafts

Four 10 day mandibular arches were grafted whole. All of them were successful. They showed the same tissues as other 10 day grafts. One graft had no teeth. Of the other three, two produced a single molariform tooth, and the other produced two molar teeth and an incisor primordium (plate 14).

Ten Day Grafts

Table II-a

10-Day Mandibular Arch Segment Grafts

Grafts placed	a	m	p
	32	32	32
Grafts retrieved			
with tooth/teeth	1 (3.1%)	14 (43.7%)	0 (0%)
without tooth/teeth	17 (53.1%)	14 (43.7%)	20 (62.5%)
total	18 (56.2%)	28 (87.5%)	20 (62.5%)
Failed grafts (failure of graft to survive)	7 (21.8%)	3 (9.3%)	8 (25%)
Anesthetic death (failure of host to survive)	7 (21.8%)	1 (3.1%)	4 (12.5%)

10 day failure rate (total) = 30:96 (31.2%)

Table II-b

Whole Arch Grafts (Hemi-mandibles)

Total	with teeth	without teeth	failed
4	3 (1 with 3 molars)	1	0

Table II-c

Number, Shape and Location of Teeth Found Developing in 10-Day Grafts

Mouse Number	a	m	p
10-1 (i)	—	1 molar	—
10-2 (i)	—	1 molar	—
10-5 (i)	—	2 molars	—
10-6 (i)	—	1 molar	—
10-6 (ii)	—	1 molar	—
10-6 (iii)	—	1 molar	—
10-8 (i)	—	2 molars	—
10-8 (ii)	—	1 molar	—
10-8 (iii)	—	1 molar	—
10-8 (iv)	1 incisor	2 molars	—
10-8 (v)	—	2 molars	—
10-9 (ii)	—	3 molars	—
10-9 (iii)	—	1 molar	—
10-9 (iv)	—	1 molar	—

Histology of 11 Day Grafts

Anterior Segment Grafts

Twenty-seven anterior segments was grafted. None were left joined in the midline. Twenty-five were retrieved. Epithelium, connective tissue, salivary gland acini, hair follicles with sebaceous glands, muscle, cartilage and bone were found in the grafts. Teeth with their associated bone and connective tissue were found in 8 anterior segment grafts. All teeth were single and incisiform (plate 15).

Middle Segment Grafts

Twenty-five of the 27 middle segment grafts were successful. The same tissue types as those found in 11 day anterior grafts were present in the middle grafts. Teeth were present in 9 cases. Five of these were single molariform teeth. Two molars were present in the other 4 grafts (plate 16).

Posterior Segment Grafts

Twenty-seven grafts were placed. Only 18 were successful. The previously described tissue types were found. Single molariform teeth were present in 3 grafts (plate 17).

Whole Arches

Three whole arch grafts were placed . Two of these produced single molariform teeth. The third graft produced only a keratin filled cyst.

Eleven Day Grafts

Table III-a

11 Day Mandibular Arch Segment Grafts

	a	m	p
Grafts placed	27	27	27
Grafts retrieved			
with tooth/teeth	8 (29.6%)	9 (33.3%)	4 (14.8%)
without tooth/teeth	17 (62.9%)	16 (59.2%)	15 (55.5%)
total	25 (92.5%)	25 (92.5%)	19 (70.3%)
Failed grafts (failure of graft to survive)	1 (3.7%)	1 (3.7%)	7 (25.9%)
Anesthetic death (failure of host to survive)	1 (3.7%)	1 (3.7%)	1 (3.7%)

11 day failure rate (total) - 12:81 (14%)

Table III-b

Whole Arch Grafts (Hemi-mandibles)

Total	with teeth	without teeth	failed
3	2	1	0

Table III-c

Number, Shape and Location of Teeth Found developing in 11-Day Grafts

Mouse Number	a	m	p
11-1 (i)	—	1 molars	—
11-1 (ii)	—	2 molars	—
11-1 a (i)	—	2 molars	—
11-2 (i)	1 incisor	—	1 molar
11-2 (iii)	—	—	1 molar
11-3 (i)	1 incisor	1 molar	—
11-3 (ii)	—	1 molar	—
11-4 (i)	—	2 molars	—
11-4 (ii)	—	—	1 molar
11-4 (iii)	1 incisor	—	—
11-5 (ii)	1 incisor	1 molar	—
11-6 (i)	1 incisor	2 molars	—
11-7 (i)	—	-	1 molar
11-7 (ii)	1 incisor	1 molar	1 molar

Wax Reconstructions

Wax reconstuctions were found to be a useful adjunct in determining the shape of teeth (plate 9b, 12b). Irregularities of shape and distortion caused by the plane of section occasionally made shape determination and even determination of number of teeth difficult. Teeth found in the grafts generally have a normal shape. Some abnormalities were found in both incisors and molars in the form of abnormal cusp shape and distortion at the apex. Reconstructions show teeth in a bony crypt often open on the coronal side in the same orientation as a normally developing tooth.

Summary of Results

9 Day

This group of grafts had the highest failure rate, 35%. Typical mandibular arch derivatives were produced by the segment grafts. Teeth, when formed, were molariform and came from the middle segment. No nine day arch produced a tooth from more than one segment.

10 day

Similar tissues were produced by 10 day grafts. Again teeth formed only from the middle segment with one exception, from an anterior graft. Some 10 day segment grafts produced more than one tooth. Only one segment from a single arch could produce teeth with one exception; that which produced an incisor from the anterior segment and a molar from the middle segment.

11 Day

The same tissue types were seen again in 11 day grafts. Teeth now developed in isolated anterior, middle or posterior segments. Some grafts produced multiple teeth. In 4 cases a single arch produced teeth from 2 of its segments (1 anterior and posterior, 3 anterior and middle). In one case a single arch was found to produce teeth from all three

of its segments.

The teeth produced from 9, 10 and 11 day mandibular arch segments were of a size and shape comparable to those developed *in vivo*. Teeth in grafts were associated with normal tissue in a normal orientaion. The tooth producing area(s) in the developing mandibular arch undergo a change in number and location between 9 and 11 days.

IV. Discussion

A. Intraocular Culture

This technique was found to be moderately successful. A total of 207/289 grafts were viable. Vascularity was established early in the anterior eye chamber (Lumsden 1978) and grafted tissues contined to grow and differentiate. Rejection phenomena were not prominent in this culture site (Fleming 1952, Lumsden 1978). Some early grafts which were left longer than 3 weeks showed rejection phenomena such as dead cells and lymphocyte infiltration. There was almost total destruction of tissue previously formed by these grafts. Longer culture times also increased the risk of infection as the enlarging graft protruded through the cornea.

B. Failed Grafts

Grafts were classed as a failure when nothing was found in the recipient's eye after 3 weeks. These grafts were possibly resorbed by the host. It is more likely that the mandibular arch segment was lost during grafting and nothing was ever present in the eye. The grafted segments were very small and translucent making it difficult to ascertain if the segment was in place or not. This was especially true of 9 day segments, thereby accounting for the higher failure rate of this group.

C. Shapes of Teeth

Teeth in grafts were usually obscured by bone and soft tissues. Infrequently they were visible to the naked eye. Lumsden (1978) noted that an advantage of intraocular grafting was that the developing teeth were visible through the transparent cornea. Lumsden, however, placed "clean" grafts of tooth primordia only. The grafts placed in this series of experiments did not contain a visible primordium, making it impossible to eliminate progenitors of other cell types without destroying the odontogenic cells. The result is that many cell types differentiated and formed mature tissues which surrounded the tooth obscuring it from view.

This factor created a problem in orientation of the graft in wax blocks for sectioning. Since the grafts must be placed in an arbitrary orientation, perfect longitudinal or cross sections cannot be obtained. The shape of a tooth as it appears in a single section may appear very distorted because of the obscure plane of section. Serial sections are required to mentally or physically reconstruct the shape of the tooth. Teeth that appeared extremely distorted when viewed under the microscope turned out to be reasonable approximations of normal teeth when reconstructed in wax.

Some deviations from normal, in the form of unusual cusp arrangements and shapes were noted. There are several possible causes. There may be a reduced capability of the clone to produce "perfect" teeth due to the trauma caused by transplantation. There may be localized death of odontoblasts or ameloblasts resulting in shape distortion. In a confined area pressure from developing cysts may have caused distortion (Fisher 1971, Lumsden 1978).

D. Root Development

Very little root formation was seen on molar teeth. More root length may have developed with longer culture periods, however grafts will not develop indefinitely in the eye. In grafts containing teeth the area of root formation, the advancing dentin margin, was often distorted. The growing edge of the tooth appeared to approach the bone, but buckled back on itself due to lack of space (plate 16).

E. Cysts

Almost all grafts, with or without teeth, had cysts. Some were very large. In some the epithelium was of the skin type complete with hair follicles and sebaceous glands.

The ubiquitous presence of cysts in intraocular grafts is well documented in the literature (Fleming 1952, Kollar 1969, Lumsden 1978). The speculated cause is placement of epithelium in the graft. In a confined space the epithelium becomes enclosed in the growing tissue and cannot shed its surface cells as it normally would. The result is an enlarging cyst

cavity. The effect of cysts on graft development is speculative. Fisher (1971) noted a correlation between massive cyst development and malformation or degeneration of cultured tooth primordia. Lumsden (1978) noted some distortion of crown form associated with cyst development. However, in the majority of his grafts cysts were separated from the tooth crowns by other tissues and did not influence crown form. Since teeth formed even in the presence of large cysts in this series of experiments, the cysts evidently did not suppress development.

F. Other Tissues Formed by the Graft

Typical mandibular arch tissues were found in grafts: epithelium (skin and mucous membrane), salivary glands, sebaceous glands, hair follicles, loose or dense connective tissue, muscle, adipose tissue, cartilage and bone. Not all of these tissues were found in all grafts. In particular, no bone was found in any 9 day anterior segments. Cartilage was found in these grafts. Ectomesenchymal cells may acquire the ability to form cartilage earlier than bone (Tyler and Hall, 1977). In this case the cells at the leading edge of the mandibular arch have not progressed through enough generations to produce bone, or are not ready to receive the bone inducing stimulus.

G. Mandibular Arch Grafts

The primary objective of this experiment was to demonstrate the presence (or lack) of a group of odontogenically competent cells in very early mandibular arches at a time when no morphologic or histologic differentiation is apparent. It is known from studies such as those of Milaire (1959) and Cohn (1957) that odontogenically competent cells are present at 12 days when the dental lamina appears with its associated accumulation of ectomesenchymal cells. Prior to this time the nature and developmental capabilities of the mandibular arch ectomesenchymal cells are open to speculation. By culturing segments of mandibular arch the present investigation has attempted to observe the progression of this group of cells from 9

days (the earliest time at which mandibular arch can be obtained and manipulated), through 10 and 11 days. The developmental capabilities of the anterior, middle and posterior segment cells of 9 10, and 11 day mandibular arches, when allowed to grow in an environment free from any cues present in the normally developing embryo, have been studied.

Theories of Tooth Development

Morphogenetic Fields

In his morphogenetic field theory of tooth development Butler (1939) hypothesises that tooth primordia exist in the developing jaw and that they are all identical until acted upon by a chemical morphogen emanating from an unknown source. All tooth primordia are created equal but, under the influence of an outside stimulus, they are induced to generate particular shapes which characterize them not merely as incisors, canine or molars, but also as specific teeth within these three classes.

Lumsden (1979) demonstrated the development in culture of the entire mouse molar dentition from an isolated 12 day M1 primordium indicating that teeth do not reguire an external chemical mediator to influence their development after 12 days. This series of experiments has gone one step further to demonstrate the development of tooth primordia from 9, 10 and 11 day mandibular arch segments. When the tissue was removed for grafting no signs of differentiation were visible, certainly no primordia were apparent. No thickening of the oral epithelium was visible under the microscope. Despite this lack of prior differentiation and unavailability in culture of normally present developmental cues, the grafts produced teeth and their supporting tissues. It is unlikely that the source of any morphogen was included in the grafts. The mandibular arches were segmented and placed separately thereby losing their original orientation and relationship to any possible field generator. Glasstone (1967) concluded that the morphogenetic field was already "mapped out" in 11 day mouse mandibular arch as she was able to culture incisors from the anterior halves of mandibular arches and molars from the posterior halves. This assumption is

difficult to disprove as it can be extended backward in development as far as need be. However, the existence of a pre-mapped out field requires that we believe in the presence of one or more field generators which cannot be defined, described or seen since no differentiation of ectomesenchyme is apparent at this time. We must also believe that all primordia exist by 9 days and that they are identical and invisible. It is therefore unlikely that any external force or chemical mediator in the form of a morphogenetic field is necessary for tooth development.

Positional Information

Wolpert's positional information model (1978) although not directly applied to teeth, would seem to suggest that cells of the mandibular arch acquire information regarding their position from the boundaries of their "field". This positional information allows them to differentiate in such a way as to develop a particular pattern. The present experimental data combined with those of Lumsden (1979) discount this theory as it applies to tooth development. Teeth continue to develop with enamel, dentin, pulp and supporting structures in a normal orientation despite having developed apart from the "field boundaries" that might have been expected to provide positional cues for differentiation. Field boundaries were not included in the grafts as the mandibular arches were segmented and each segment placed separately.

There appears to be no evidence to support an hypothesis of tooth development that requires an external influence for histodifferentiation and morphodifferentiation. The evidence from this series of experiments and those of Lumsden(1979) indicates that odontogenic tissue is inherently competent from an early stage, at least as early as 9 days. The clonal theory of tooth development (Osborn 1978a) is supported by the weight of experimental evidence. According to this theory control of odontogenic differentiation exists within the clone of odontogenic cells. Osborn's theory requires that a clone of competent cells (dental clone mesenchyme) be initiated at some point in embryonic development (fig. 10). The dental clone mesenchyme (D.C.M.) gives rise to two other

clones: incisor clone mesenchyme (I.C.M.) and posterior clone mesenchyme (P.C.M.). The P.C.M. then gives rise to canine clone mesenchyme (C.C.M.) and molar clone mesenchyme (M.C.M.). These clones then grow and progress to bud off primordia sequentially (fig. 8). In this case all primordia are created unequal and the shape gradient present in each class of teeth is a reflection of the cell ancestry of their respective primordia.

The existence of these clones can only be postulated as these cells are not visibly different from their neighbors. We must infer the presence of a clone from the ability of the apparently undifferentiated cells to form teeth when grown in culture. Lumsden (1979) has previously demonstrated the development in culture of the entire mouse molar class from the first initiated molar primordium. We have attempted to follow the development of these cells as well as those of the incisor clone from an earlier stage.

9 Days

Our results indicate that 9 day mandibular arches contain cells capable of producing teeth without external influences. The tooth producing area of a 9 day arch was confined to the middle segment and was capable of producing single (7 out of 8) and occasionally multiple (1 out of 8) molars. In no case, either segmented or whole arches, did 3 molars develop. It is possible that under ideal grafting and culture conditions an incisor and 3 molars would be produced. However *in utero* conditions cannot be duplicated entirely and technique is difficult to perfect when working with such tiny fragile grafts. It is also possible that the 9 day molar clone has not yet achieved the capacity to form a third molar. Trauma caused during isolation and placement of the graft may have irreparably damaged the ability of the clone to produce all teeth. Disruption of the cell cycle or inability to completely recover from transplantation would result in some loss of competence of the clone and a decreased number of teeth in the graft.

When cultured whole (hemi-mandible) one 9 day mandibular arch produced an incisor plus two molars. Apparently the ability to autonomously produce an incisor and molars is

present very early. At 9 days there is very little extension of ectomesenchyme across the midline. A thin epithelial tube extends across the midline with a small central core of mesenchymal cells. The presence of an incisor in a graft from only one side indicates that no interplay of right and left sides is required to produce an incisor. This result was observed in only one instance and may have been an isolated event rather than a general trend.

Anterior and posterior segments of 9 day mandibular arch failed to produce teeth. Posterior segments were viable and survived to produce other mandibular arch tissues. Anterior segments had difficulty surviving possibly due to their small size relative to older anterior segments and lack of a significant ectomesenchymal component. When left joined across the midline, the a-a units survived and produced appropriate mandibular arch tissues, but not teeth or bone.

In the 9 day mandibular arch the odontogenic clone exists as a single group of cells occupying the middle segment of the arch. In culture these cells have the inherent ability to form one or more molars. The cells grafted as anterior and posterior segments were members of clones other than the odontogenic clones.

10 Days

At 10 days the graft results are similar in that odontogenic cells appear to be still centred in the middle segment. In only one instance did any other segment produce a tooth. An anterior segment developed an incisor, while the middle segment of the same arch produced a molar.

The anterior segments of 10 day arches were now viable alone and did not require the support of the other side. This was perhaps due to the increased population of ectomesenchymal cells in this area. When few cells are present, as in a 9 day arch, the proportion damaged by surgery is much greater and the chance of survival is decreased. Tissues found in 10 day anterior segment grafts were the same as those produced by 9 day a-a segments except that bone could now be formed by these grafts and, in one instance, a tooth. Posterior

segments produced mandibular arch derivatives but no teeth.

In the 10 day mandibular arch the odontogenic clone appears to be still single and located in the middle segment. When isolated the clone produces molars. The grafts from one 10 day arch indicated the presence of two distinct odontogenic groups, one capable of producing an incisor, the other producing a molar.

11 Day

Normal mandibular arch tissues (bone, cartilage and soft tissues) were present in 11 day grafts as they were in grafts of earlier mandibular arch segments.

Eleven day graft results show a change in that teeth are still being produced from the middle segment (molars) (33.3%), but also from the anterior segment with increasing frequency (29.6%). Teeth are now found in the posterior segment for the first time (14.8%).

6 out of 14 arches grafted as segments produced both an incisor and one or more molars indicating the presence of two odontogenic clones. The single initial molar producing clone present at 9 days, now appears at 11 days as two separate and distinct areas of odontogenic competence; an anterior area producing an incisor, a more posterior area producing the molars. The two groups appear to be increasingly far apart as the molar group is occasionally detected in the posterior rather than the middle segment.

The origin of the second clone is not readily apparent. One possibility is that a group of previously unspecialized cells in the anterior area achieves odontogenic competence through an unknown internal or external stimulus. We have already questioned the need for external stimuli in tooth development. The presence of "unspecialized" cells is unlikely as well, since all cells at any given time are members of a specific clade and have limited options available (fig. 10). These factors argue against a separate development for the second clone. However, there is still the possibility of a separate development of the anterior clone in the same way as the initial clone was derived from branchial arch ectomesenchyme, but at a later time.

The most likely possibility is that we are observing the division from D.C.M. to I.C.M. and P.C.M. as postulated by Osborn (1978b). The single clone seen in 9 and 10 day arches represents the D.C.M. described by Osborn. By 11 days it has progressed to the point where it can now form the two clones responsible for incisor formation (I.C.M.) and the posterior dentition (P.C.M.).

The Missing Canine

According to Osborn's cladistic sequence of development in the generalized mammal (Osborn 1978b), the P.C.M. would now progress to form C.C.M. and M.C.M. This state of development is never achieved in mice, which have no canines. Only two dentally competent areas are found in grafts of mouse mandibular arch segments. In a single case a surgically segmented 11 day arch produced a tooth from each of the three segments. The anterior segment produced a single incisor while the middle and posterior segments each developed a single molar. Since this result was seen only once, and produced no extra, unusual or "canine" teeth, it probably does not indicate the development of a third clone, but rather the surgical division of the P.C.M. by the cut through the arch prior to grafting. Dentally competent cells of the same clone were present in both middle and posterior segments.

The inability of mouse mandibular arch to form C.C.M. must not be entirely due to lack of space because a long tooth-free diastema separates the incisors from the molars The cause of the missing canine is more complex, involving a number of integrated factors. The P.C.M. may not undergo the required number of cell divisions necessary to achieve competence to form C.C.M. and M.C.M., due either to lack of space or time. There may be an incoordination of cell divisions and inductive stimuli (internal or external) such that the C.C.M. is never allowed the opportunity to develop. Mice have no canines because it is genetically not possible for them to create the exact set of circumstances that produce a C.C.M., hence no canine.

V. Summary

1. Segmented 9 day mandibular arches demonstrate only one area of odontogenic competence. This clone is in the middle of the developing arch and invariably produces a molar tooth (or teeth).

2. Ten day mandibular arch typically follows the same pattern as 9 day tissue in that the only segment capable of forming teeth is the middle one. Again one or two molars are formed. There is some early indication of the formation of 2 tooth producing areas as one mandibular arch produced an incisor from the anterior segment and 2 molars from the middle segment.

3. Eleven day mandibular arches yield two tooth producing groups of cells: an incisor producing group in the anterior segment plus a molar producing group in the middle or posterior segment.

These data support the clonal theory of tooth development as suggested by Osborn (1978a). The presence of odontogenic clones and their development from 9 to 11 days has been demonstrated. The isolated development of these cell groups in culture produced normal teeth in a pattern typical of the mouse apparently without benefit of external controls. No canines were formed and no evidence was found to suggest the existence of a third (canine producing) odontogenic clone in the mouse.

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Appendix 1. Figures

Fig. 1 Transverse sections of developing neural plate, beneath which is the notochord in the midline, illustrating the formation of the neural tube and neural crest. (Black areas indicate neural crest.) As the neural plate folds during development neural crest cells migrate away from the developing tube.

Fig.2 (Sagittal section of a 8 day mouse embryo) Development of the foregut pocket illustrates the developing buccopharyngeal membrane consisting of ectoderm and endoderm without intervening mesoderm. (from Theiler 1978)

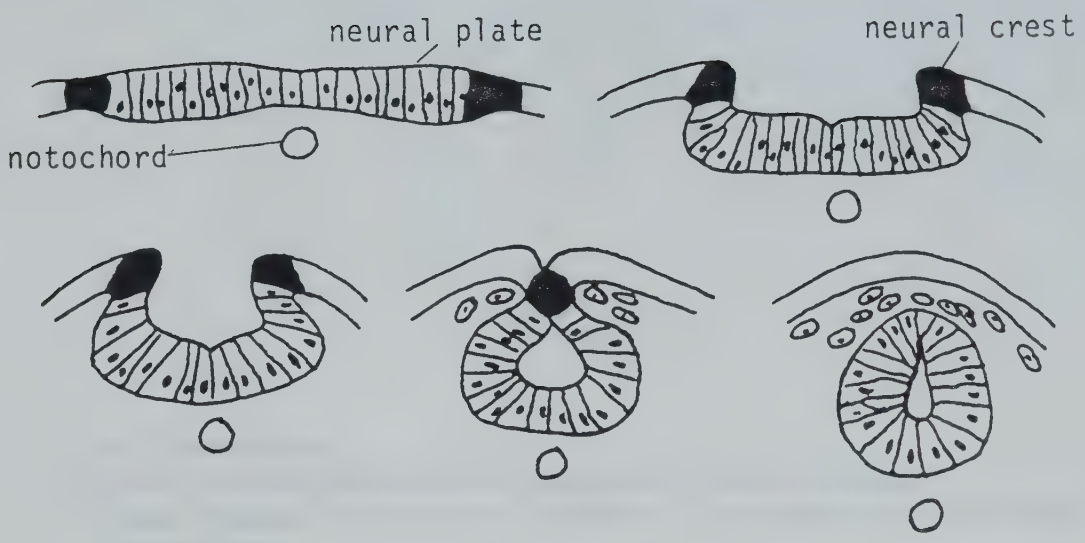

neural plate
neural crest
notochord

fig. 1

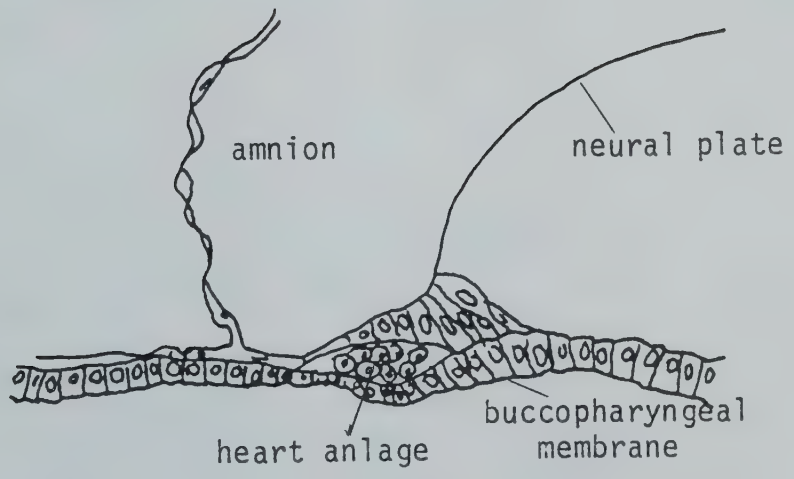

amnion
neural plate
buccopharyngeal membrane
heart anlage

fig. 2

Fig. 3 8 day (6 somites) mouse embryo and its membranes, in the sagittal plane, showing dorsal flexure.

Fig.4 Tooth development
Stages in the development of the lower central incisor (human).
1. Dental lamina. An extension of epithelium is growing into the underlying ectomesenchyme..
2. Cap stage. Ectomesenchymal cells are clumping beneath the cap of ectoderm.
3. Early bell stage. The dental lamina extends to the permanent primordium.
4. Advanced bell stage. Dentin deposition begins. The dental lamina is breaking down and a crypt of alveolar bone is developing to surround the tooth.
5. Crown near completion. Enamel and dentin are laid down. The permanent tooth is now in in the bell stage.
6. Root formation and eruption of the deciduous tooth. Enamel and dentin are forming in the permanent tooth.

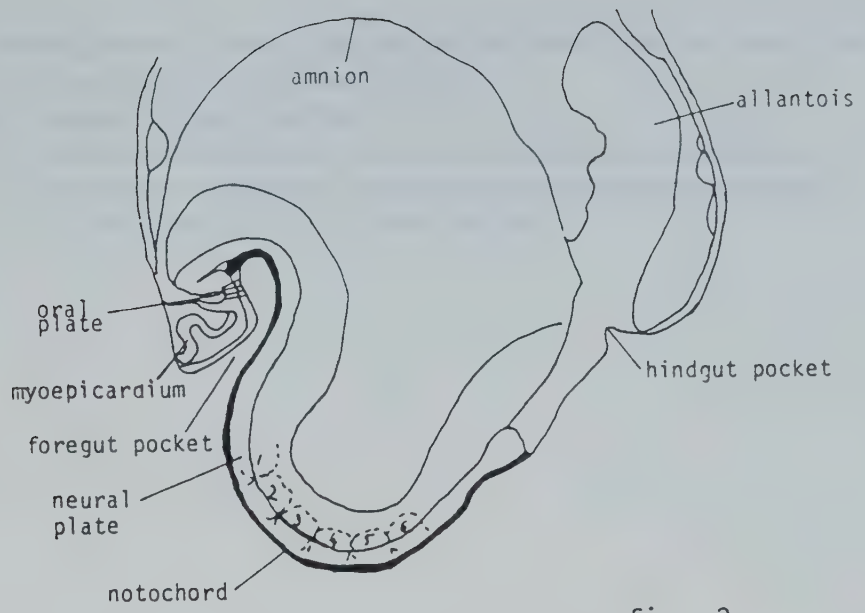

amnion
allantois
oral plate
myoepicardium
foregut pocket
neural plate
notochord
hindgut pocket

fig. 3

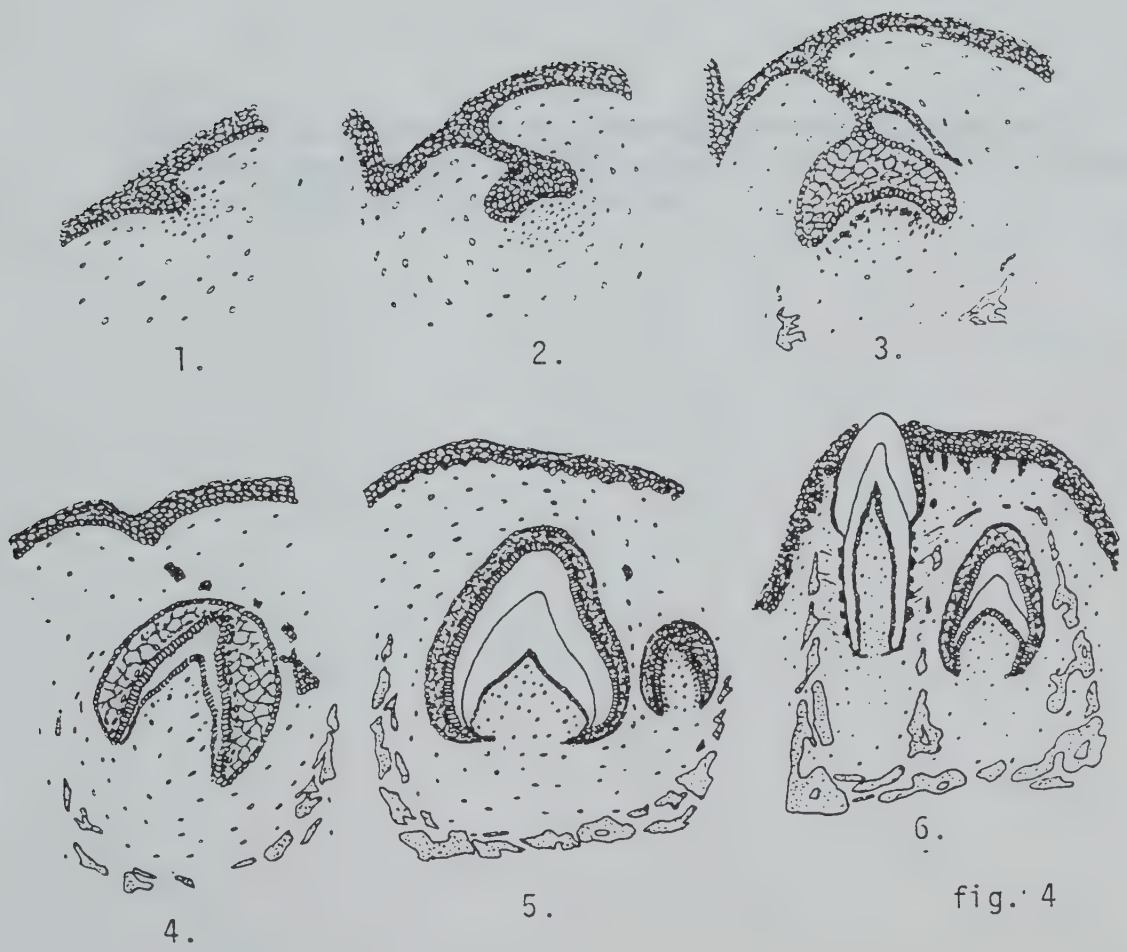

1.
2.
3.
4.
5.
6.

fig. 4

Fig. 5 Fate map showing zones of amphibian neural crest seen from the dorsal side as defined by Horstadius. (from Horstadius 1950)
Zone 1 and 2: unable to form catrilage.
Zone 3 and 4: supplies the cells that form the mandibular arch skeleton.
Zone 5 to 7: supplies the cells that form the hyoid and branchial skeletons.
Zone 8: trunk neural crest, unable to form cartilage.

Fig. 6
Similar fate map showing zones of amphibian neural crest as defined by Chibon. (from Chibon 1967) Cells that originate in the area 30 to 100 degrees from the midline form the dental papilla.

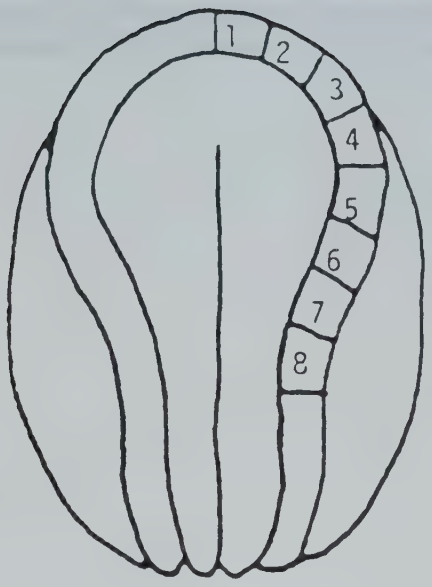


fig. 5

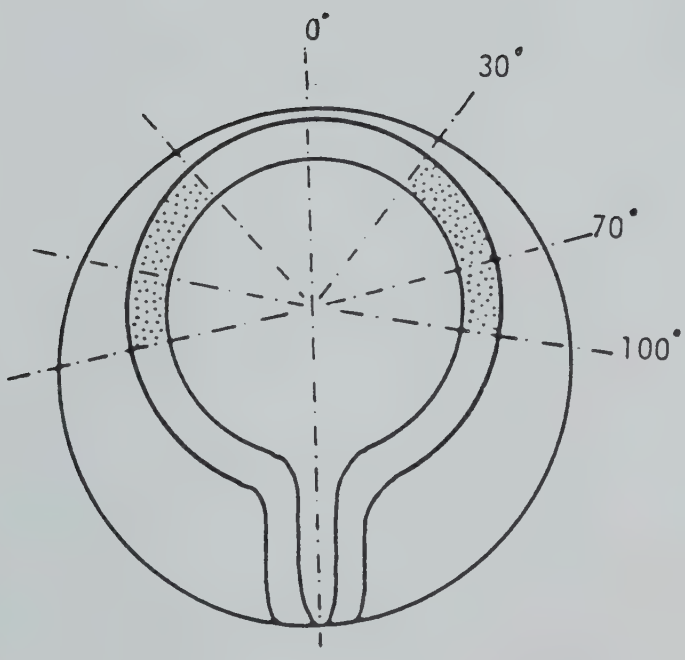


fig. 6

Fig. 7 The morhogenetic field gradient model for the development of the eutherian dentition. 11 identical primordia (a) become 3 incisors, 1 canine and 7 postcanines (c) through the influence on them of varying levels of 3 different morphogenetic field substances (b). (from Butler 1939)

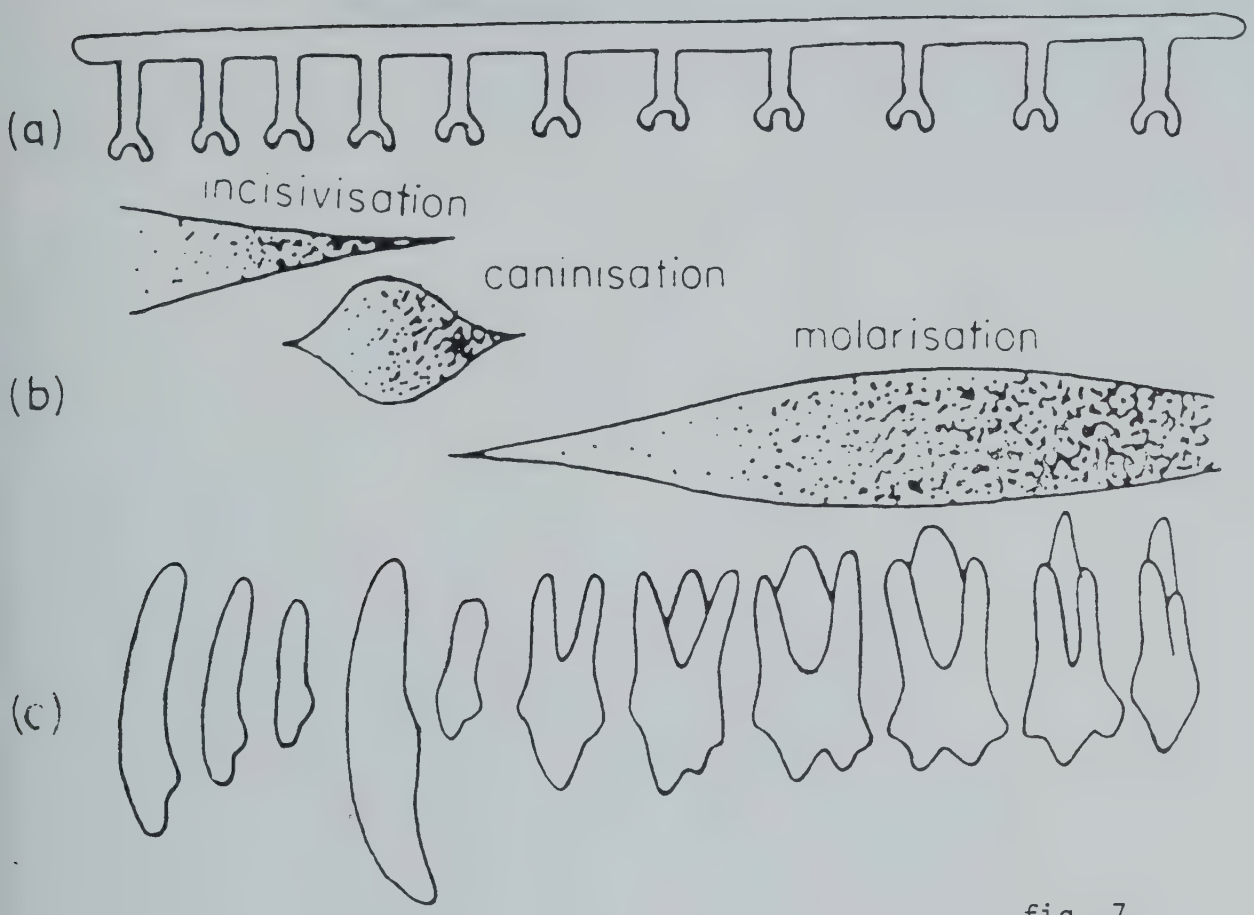


fig. 7

Fig. 8 The lower jaw of an embryo mammal contains three clones represented by three small rectangles (A). Though growth the clones become able to initiate tooth primordia. For each clone the initial primordium is the stem progenitor (B), shown in black. Continued anterior, posterior and inferior interstitial growth allows the clones to bud off the remaining dentition (C,D), including the replacement teeth (ovals). Three tooth classes, incisor, canine and molar are generated (E).

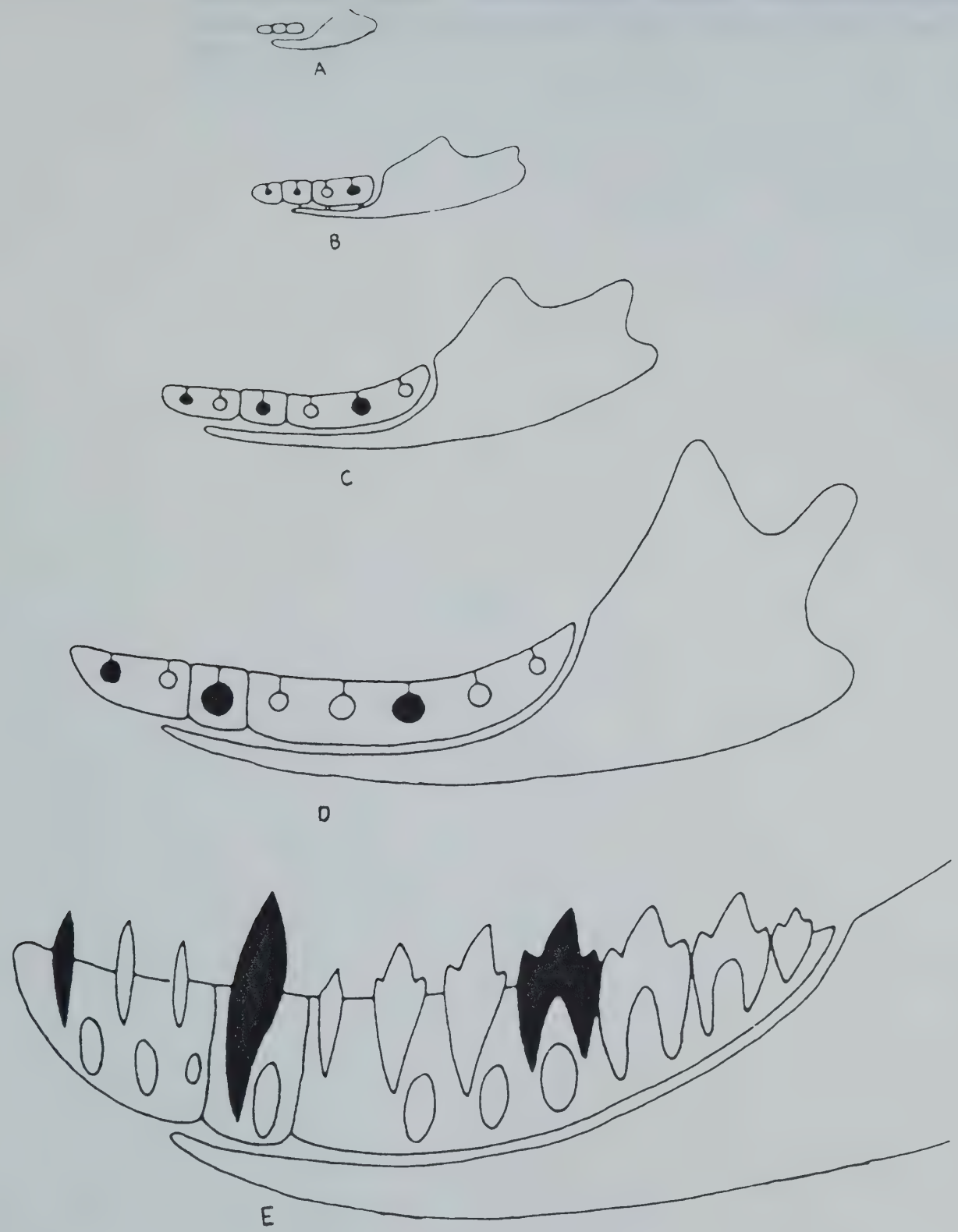


fig. 8

Fig. 9 Dorsal view of a 10.5 day mouse mandibular arch indicating presumptive tooth forming areas (stippled). The anterior area produced an incisor when developed in culture. The posterior area produced a molar or molars. (from Lumsden 1984)

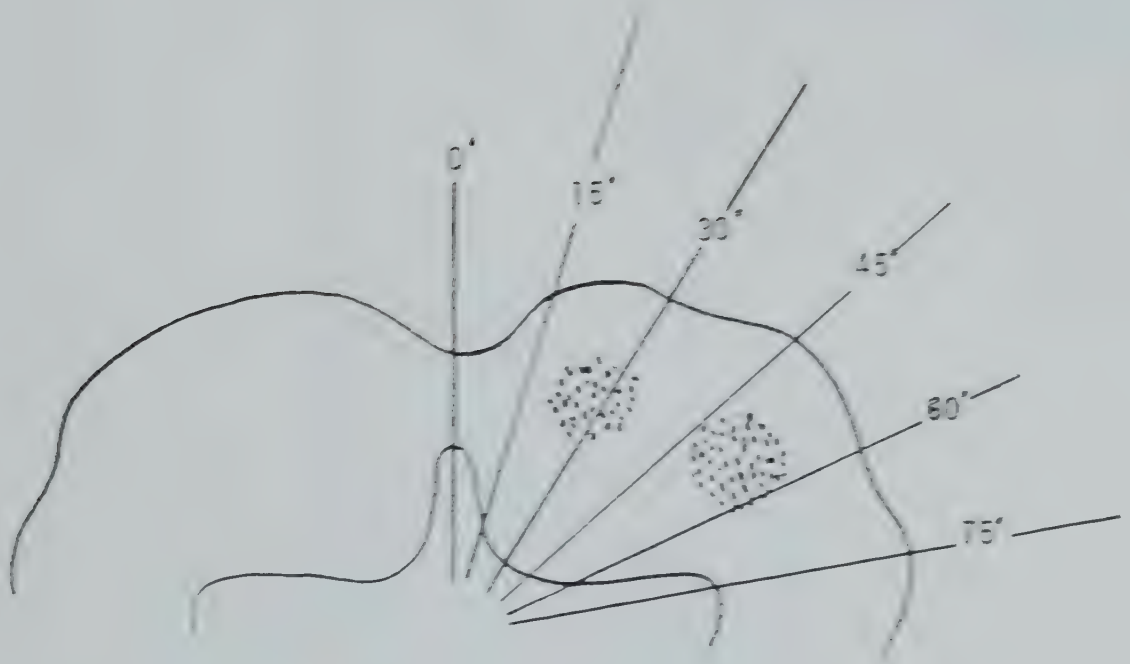


fig. 3

Fig. 10 A simplified cladogram to illustrate the derivation of the 3 dental clones and the basal clone from the neural crest. The inductive mechanisms that stimulate these differentiations are unknown. The roles of pharyngeal endoderm and mandibular arch epithelium in differentiation of cartilage and bone have been previously discussed. I.C.M. (incisor clone mesnchyme)
P.C.M. (posterior clone mesenchyme)
C.C.M. (canine clone mesenchyme)
M.C.M. (molar clone mesenchyme)

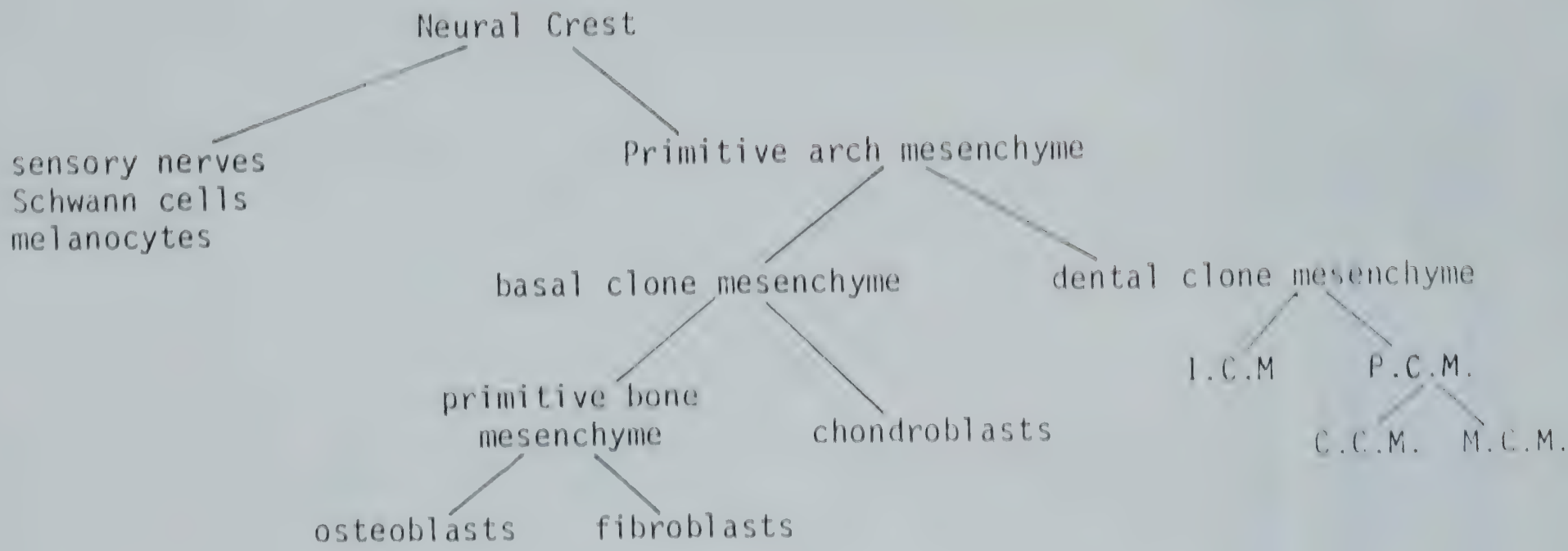
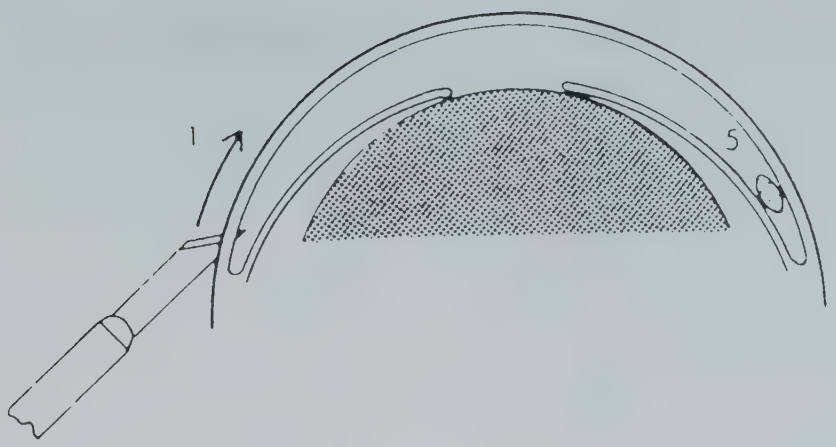

Neural Crest
sensory nerves
Schwann cells
melanocytes
Primitive arch mesenchyme
basal clone mesenchyme
dental clone mesenchyme
primitive bone mesenchyme
chondroblasts
I.C.M
P.C.M.
C.C.M.
M.C.M.
osteoblasts
fibroblasts

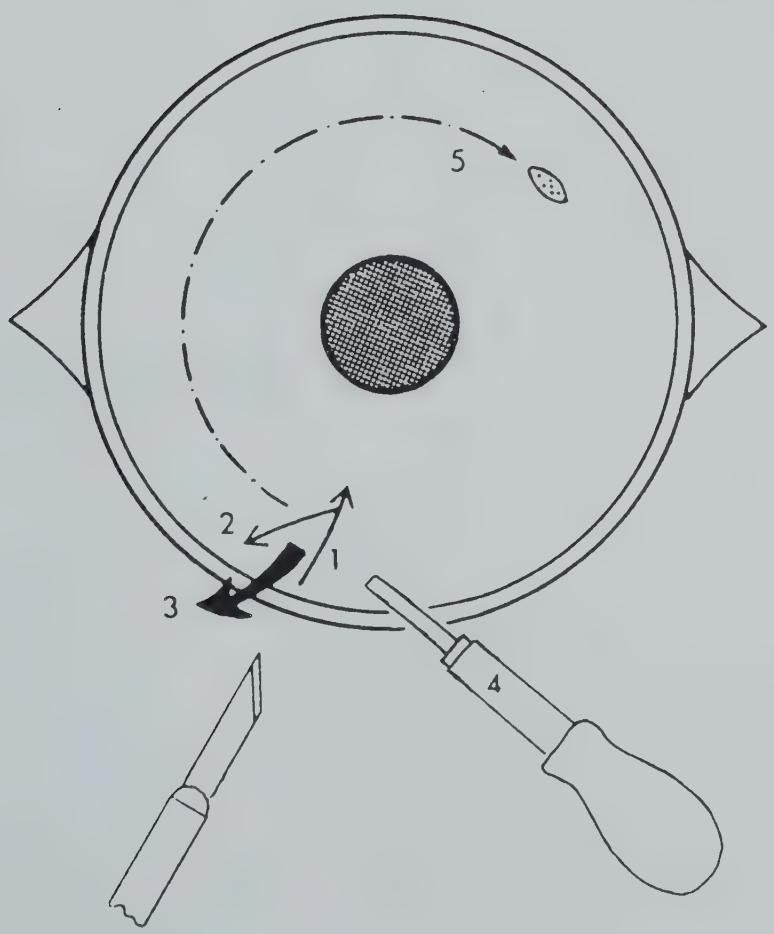
fig. 10

Fig. 11 Diagram of the adult mouse eye showing the sequence of operations in the grafting technique.
A Vertical section
B Plan view

1. First incision at the limbus, directed toward the pupil (stippled).
2. Second incision directed back toward the limbus with ophthalmic micro-spring scissors.
3. Reflection of the corneal flap.
4. Insertion of the graft using a fine glass tube and rubber bulb.
5. Implant moved to the side opposite the incision and secured in the iridiocorneal angle.



A



B

fig. 11

Fig. 12 9 day mouse embryo head

Fig. 13 10 day mouse embryo head

Fig. 14 11 day mouse embryo head

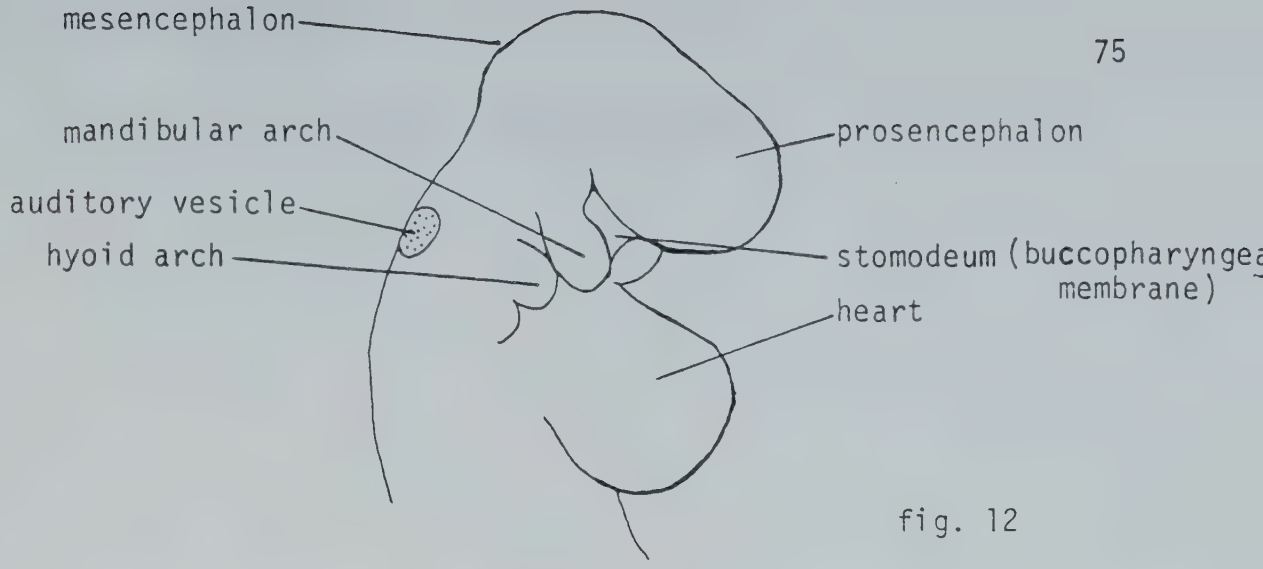


fig. 12

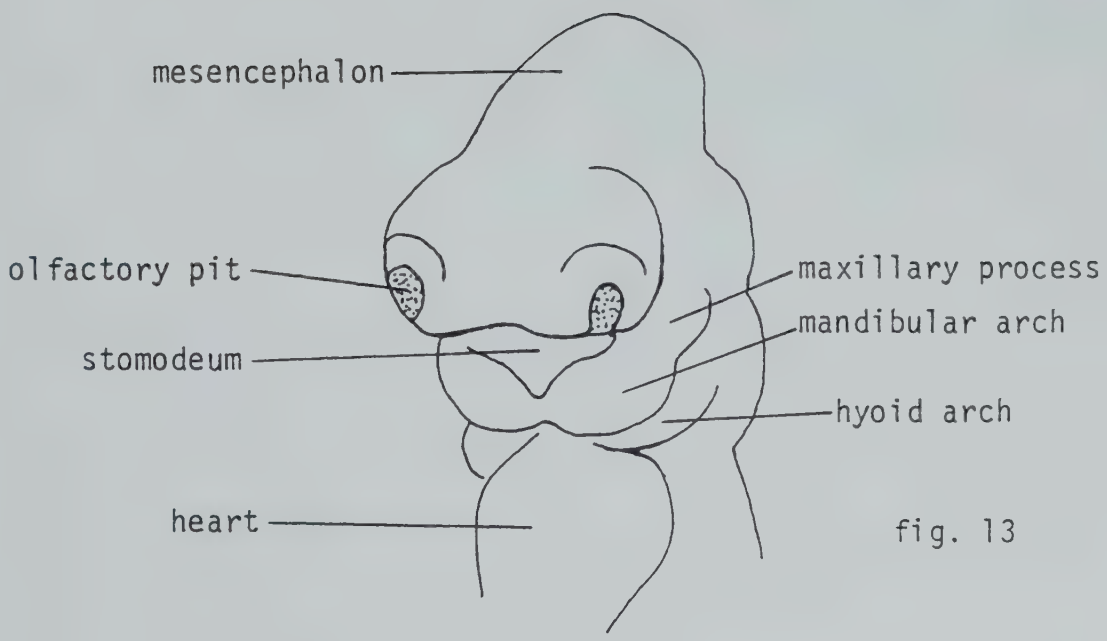


fig. 13

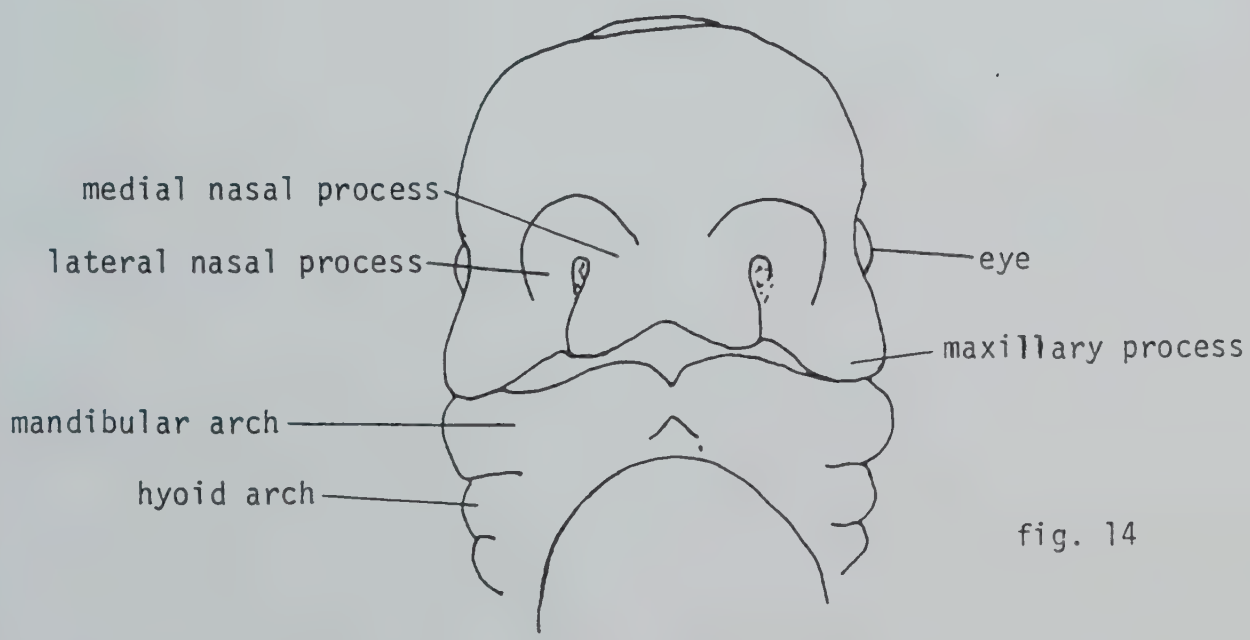


fig. 14

Appendix 2. Photographic Plates

Plate 1

9 day mouse embryo head (parasagittal section, hematoxylin and eosin stain, magnification x60)

1. mandibular arch
2. fronto-nasal process
3. heart
4. optic stalk

2
4
1
3

Plate 2

9 day mandibular arch (parasagittal section, hematoxylin and eosin stain, magnification x1500)

a. Oral surface: The epithelial layer is 1-2 cells thick of cuboidal/columnar cells.
b.external surface: The epithelial layer is thinner and the cells are flattened.

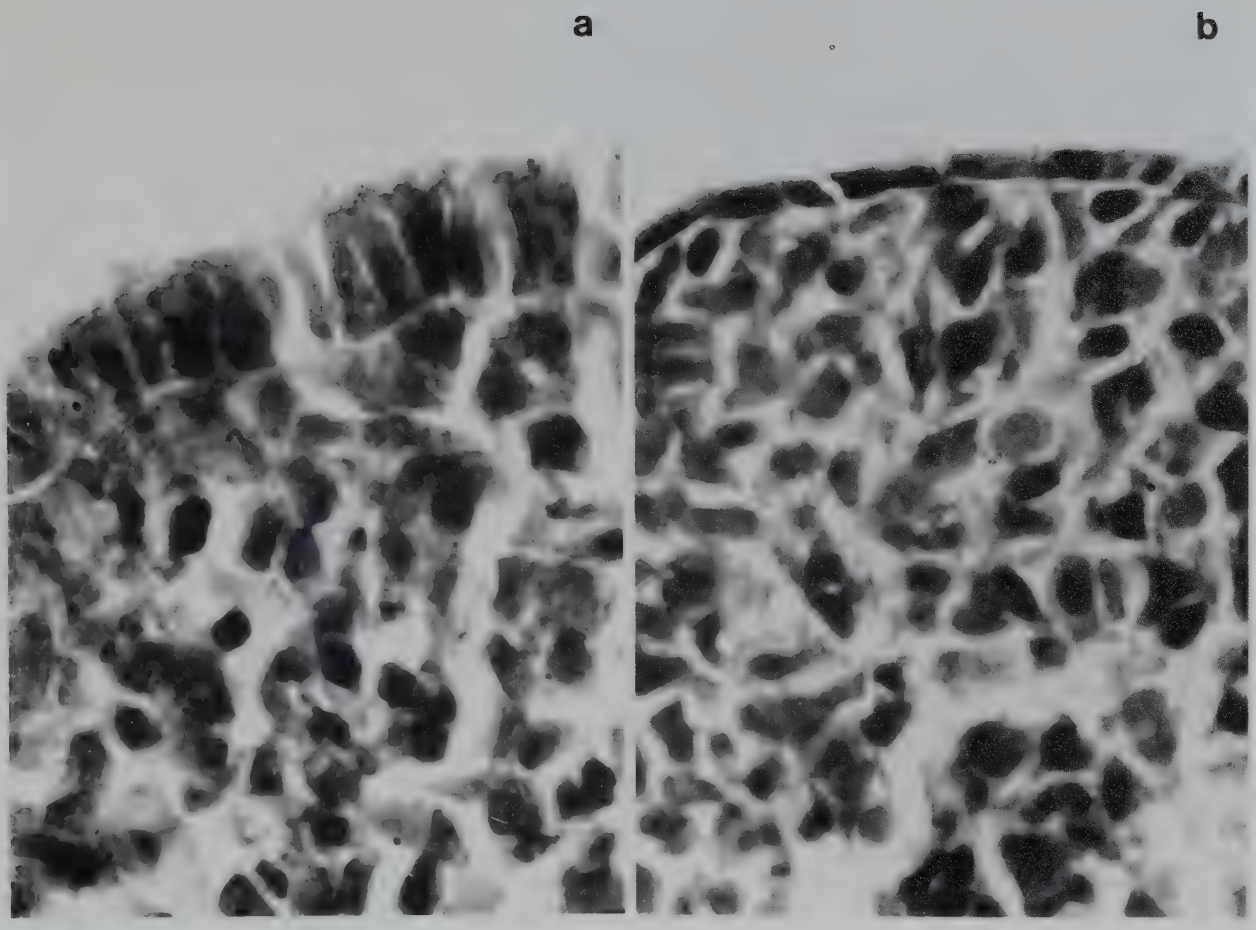

a
b

Plate 3

10 day mouse embryo head (parasagittal section, hematoxylin and eosin stain, magnification x60)

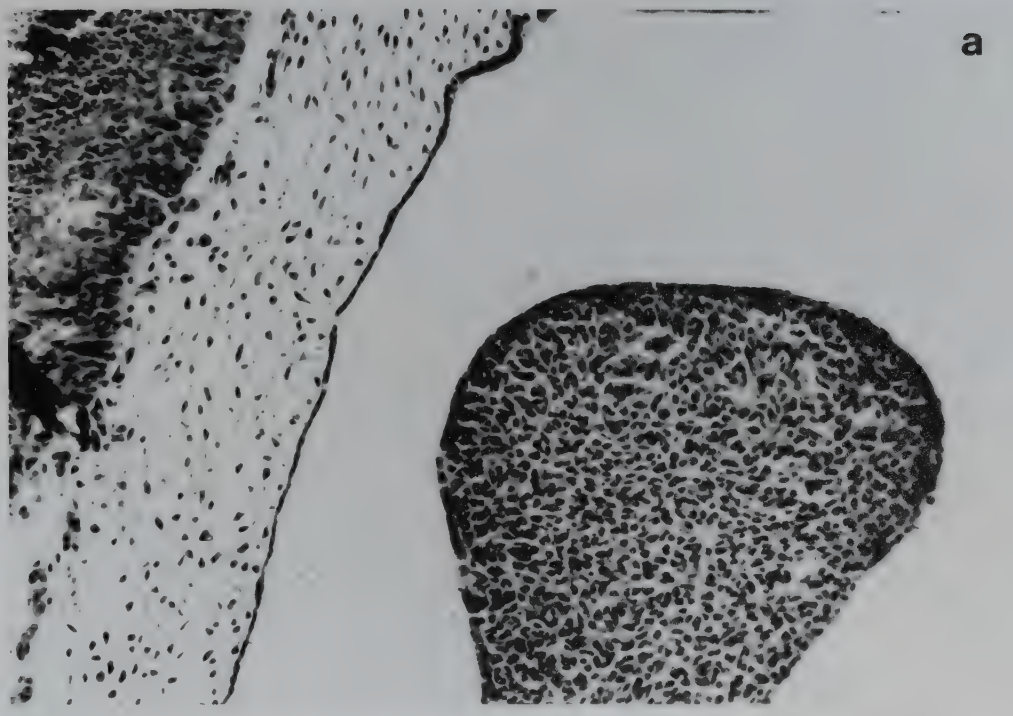
1. mandibular arch
2. hyoid arch
3. nasal pit
4. optic stalk

3
4
1
2

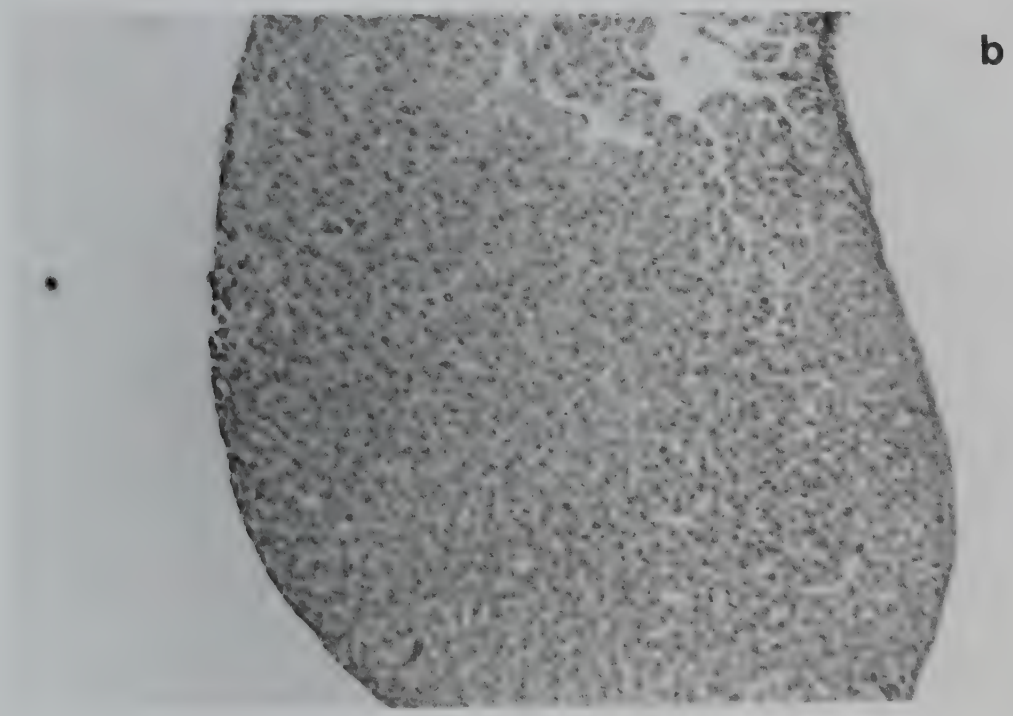
Plate 4

10 day (hematoxylin and eosin stain)

a. Mandibular arch of the embryo in plate 3. (magnification x200) The oral surface epithelium is thicker than that covering the external surface.
b.Transverse section of the mandibular arch after removal from embryo; ectomesenchymal cells show no morphologic evidence of differentiation. (magnification x400)



a



b

Plate 5

11 day mouse embryo head (frontal section, hematoxylin and eosin stain, magnification x60)

1. mandibular arch
2. maxillary arch
3. eye
4. diencephalon
5. heart

4
3
2
1
5

Plate 6

11 day mandibular arch

a. Whole arch surgically removed from the embryo and cut approximately in transverse section (hematoxylin and eosin stain, magnification x50). The ectomesenchymal cells are similar in appearance to the earlier stages, however there is some indication of accumulation of cells in the anterior and posterior areas.
b. 11 day mandibular arch (Linder's nerve stain, magnification x1500) The nerve appears black and somewhat reticular.

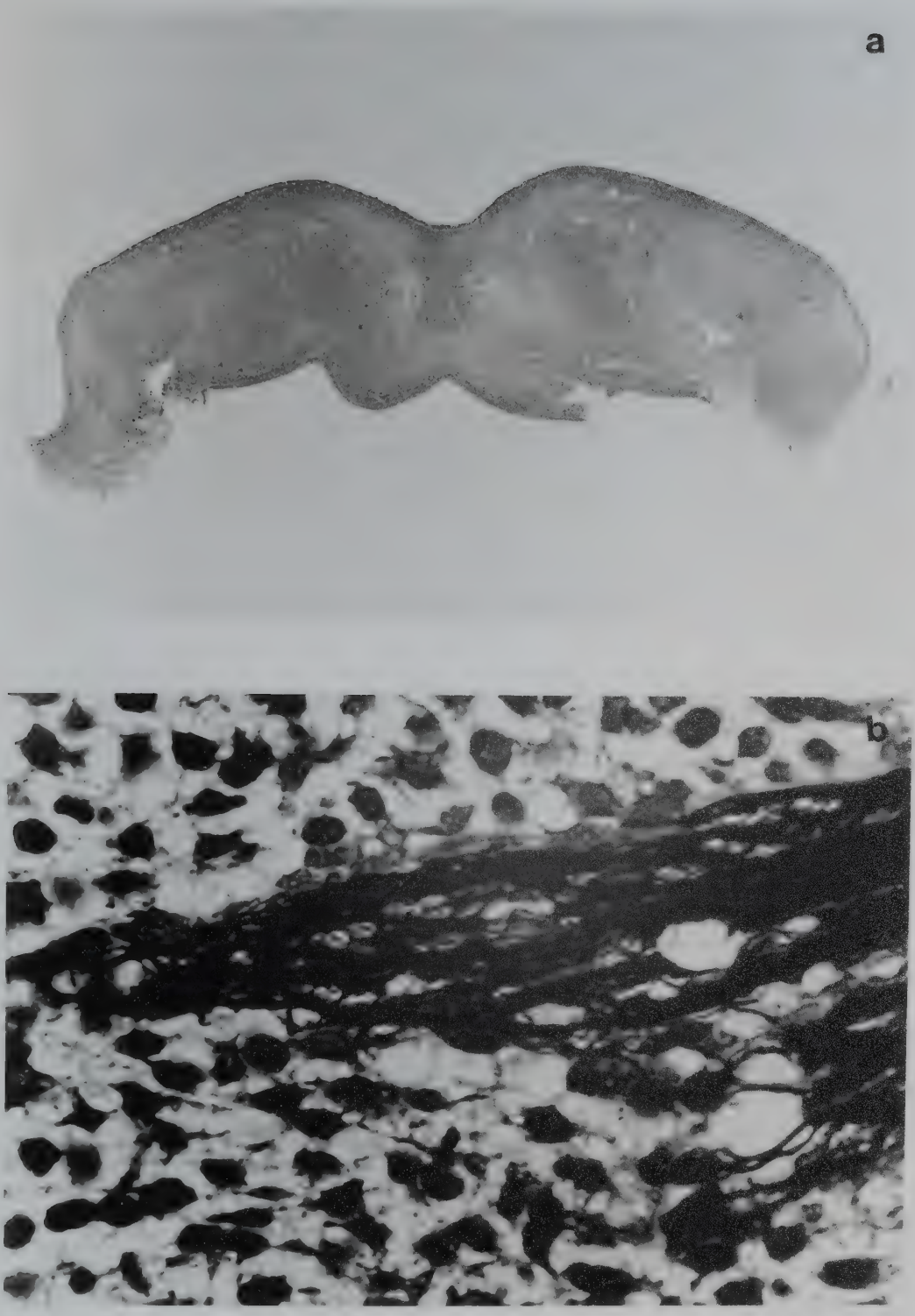

a
b

Plate 7

12 day mandibular arch (parasagittal section, hematoxylin and eosin stain, magnification x200) (anterior is to the right.)
1. Epithelial component of tooth primordium (dental lamina stage, see figure 4).
2.ectomesenchymal component.
This plane of section shows the epithelial thickening on the mandibular arch only.

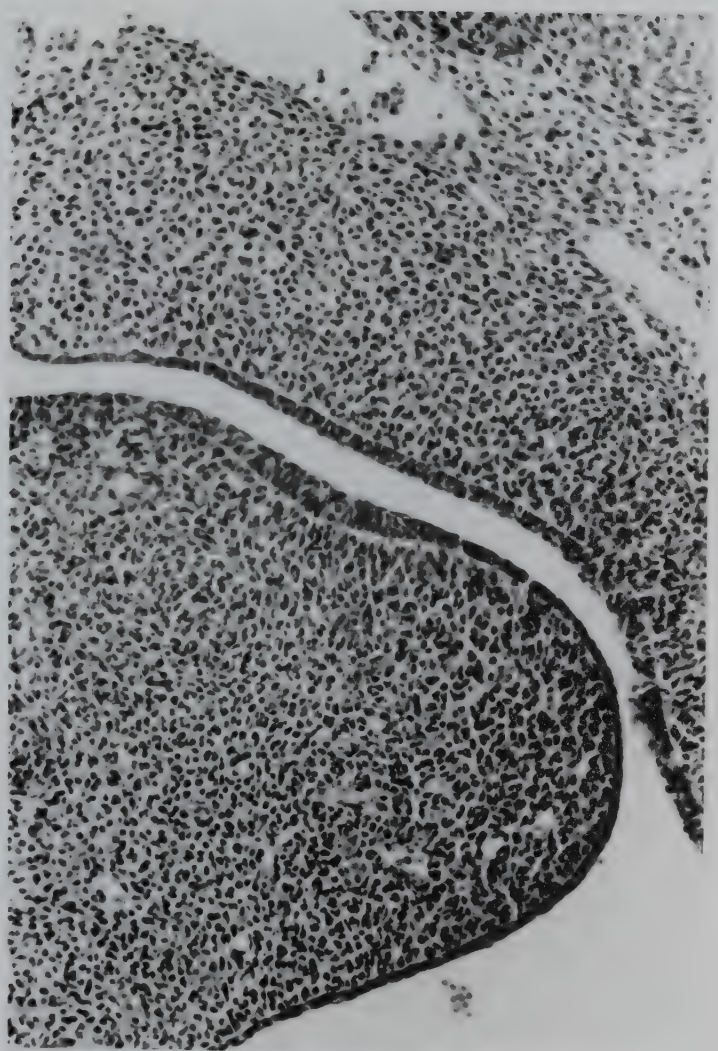


Plate 8

9 day anterior-anterior segment graft (hematoxylin and eosin stain, magnification x60)

1. cyst cavities with epithelial lining
2. salivary gland, identified by the similar appearance to that seen in adult tissue.
3. sebaceous gland, also identified by its similarity to adult tissue.

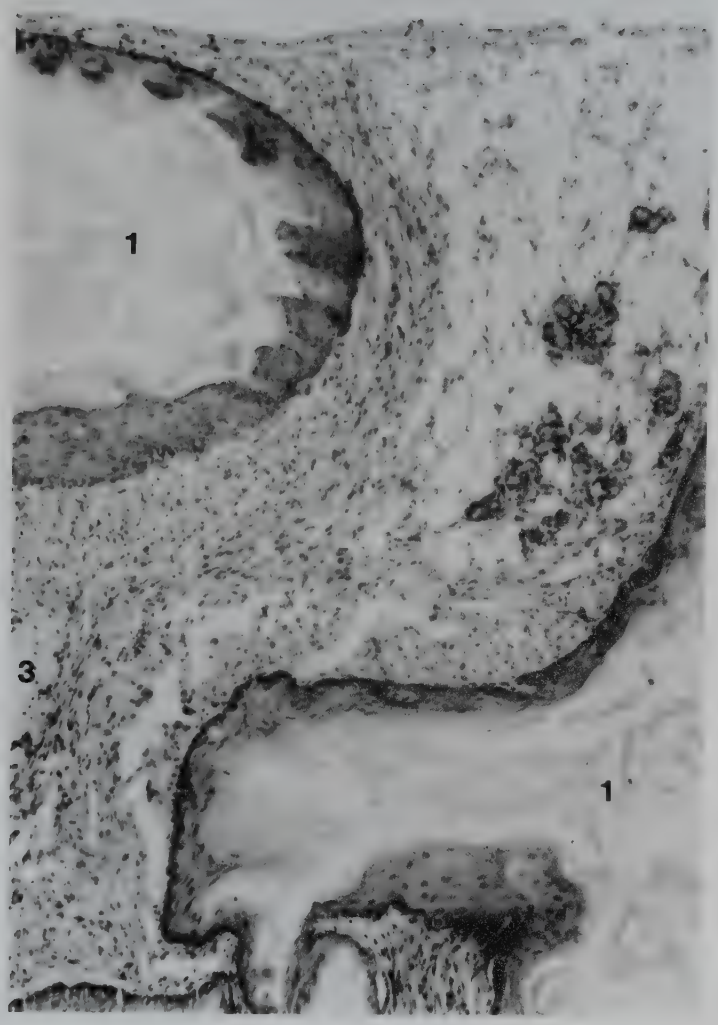
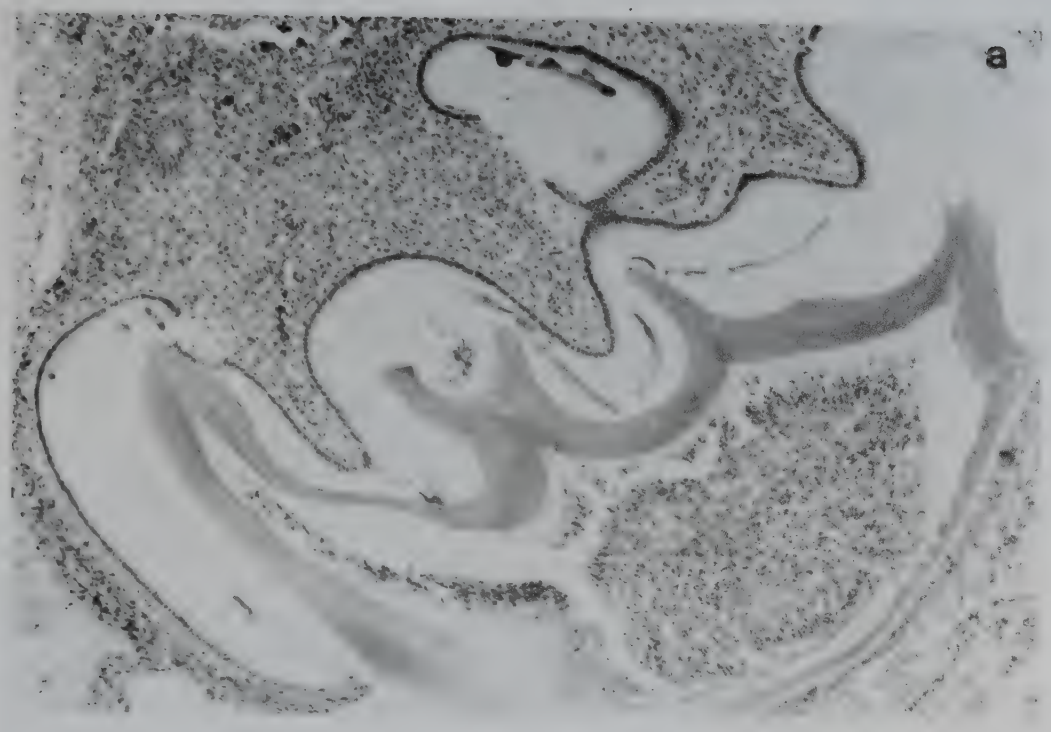

1
3
1

Plate 9

9 day (demineralized section, hematoxylin and eosin stain, magnification x200)

a. Middle segment graft, showing a molar primordium with advanced crown development and no root development. The primordium is surrounded by a bony crypt (not shown) but separated from it by a dental follicle.The folded dentin is surrounded by a space which was occupied by enamel. This enamel is almost totally lost during demineralization.
b. A part of a wax model of the tissue, a section of which is shown in a. The hard tissues (including enamel) lie in the middle. The pulp chamber of the tooth is open below. The tooth is surrounded by bone except around the opening of the pulp.


a

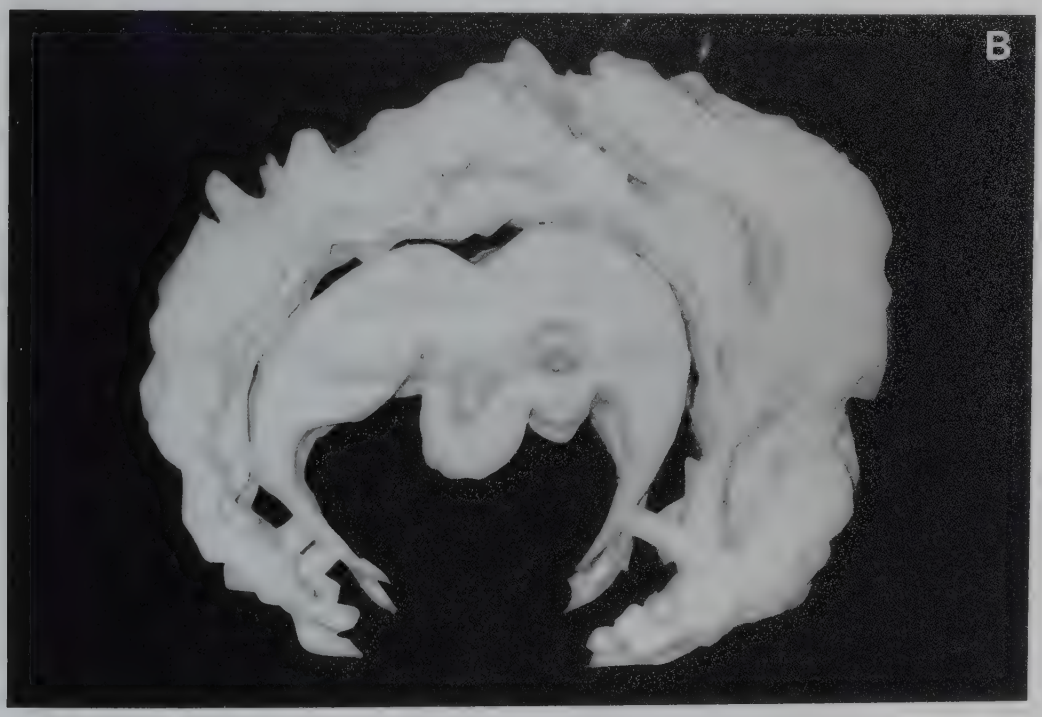

B

Plate 10
9 day whole arch graft (hematoxylin and eosin stain, magnification x60)

1. incisor primordium, identified by lack of folding in the enamel surface.
2. molar primordium, the future enamel/dentin interface is folded.
3. bone

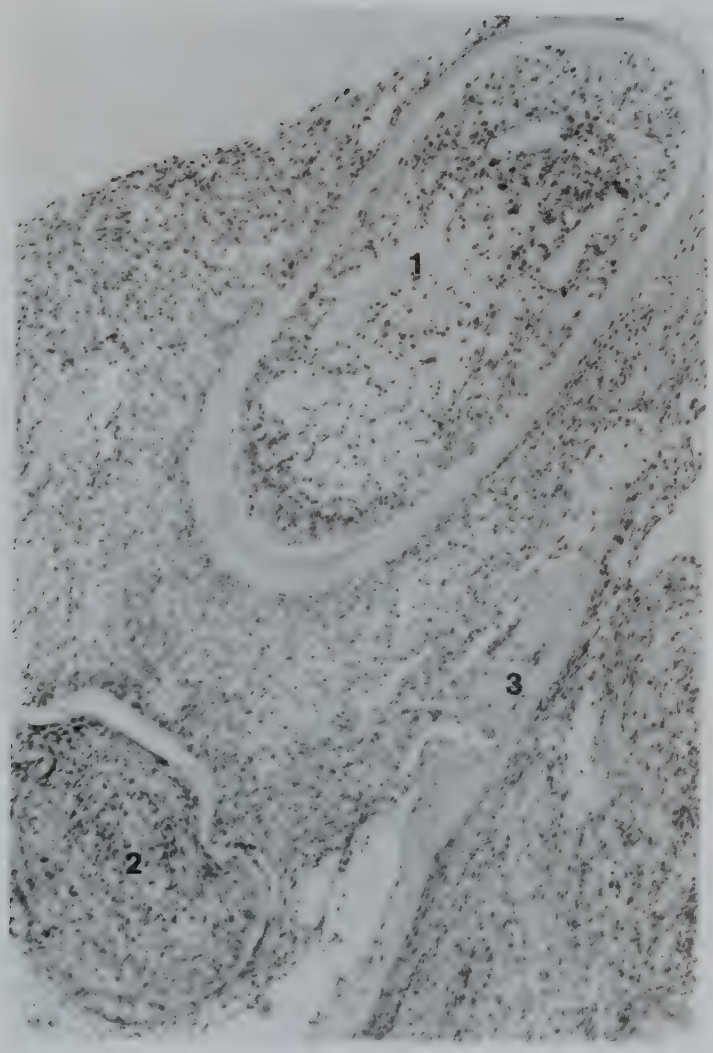

1
2
3

Plate 11

10 day anterior segment graft (hematoxylin and eosin stain, magnification x60)

Serial sections revealed an absence of teeth in this graft.
1. cyst cavity
2. bone
3. cartilage

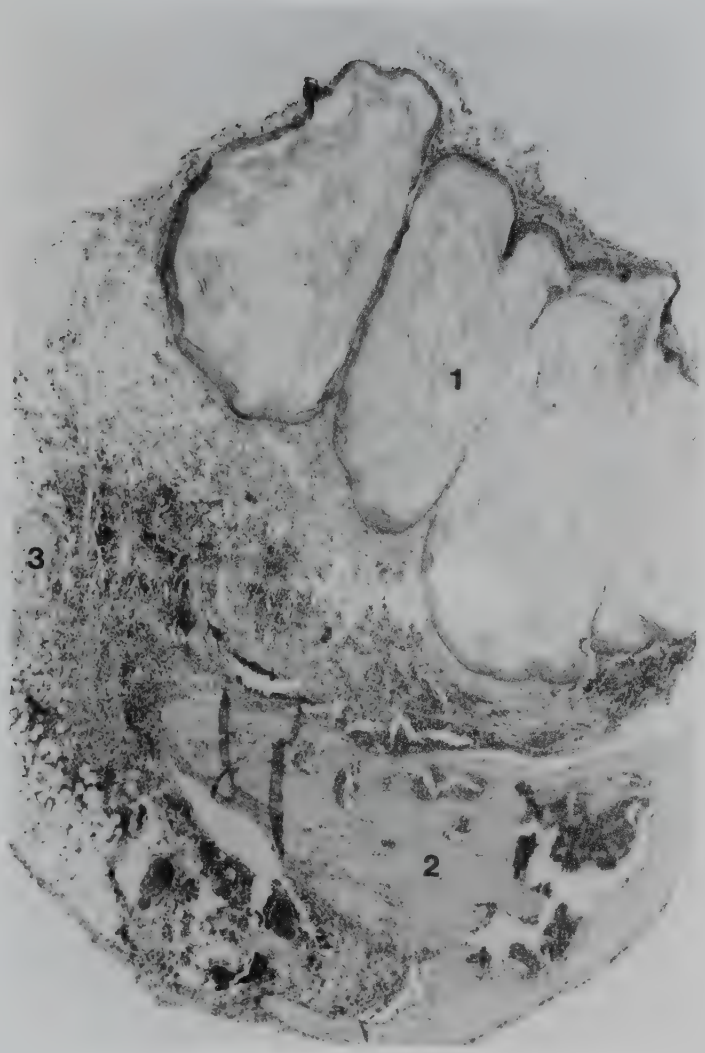
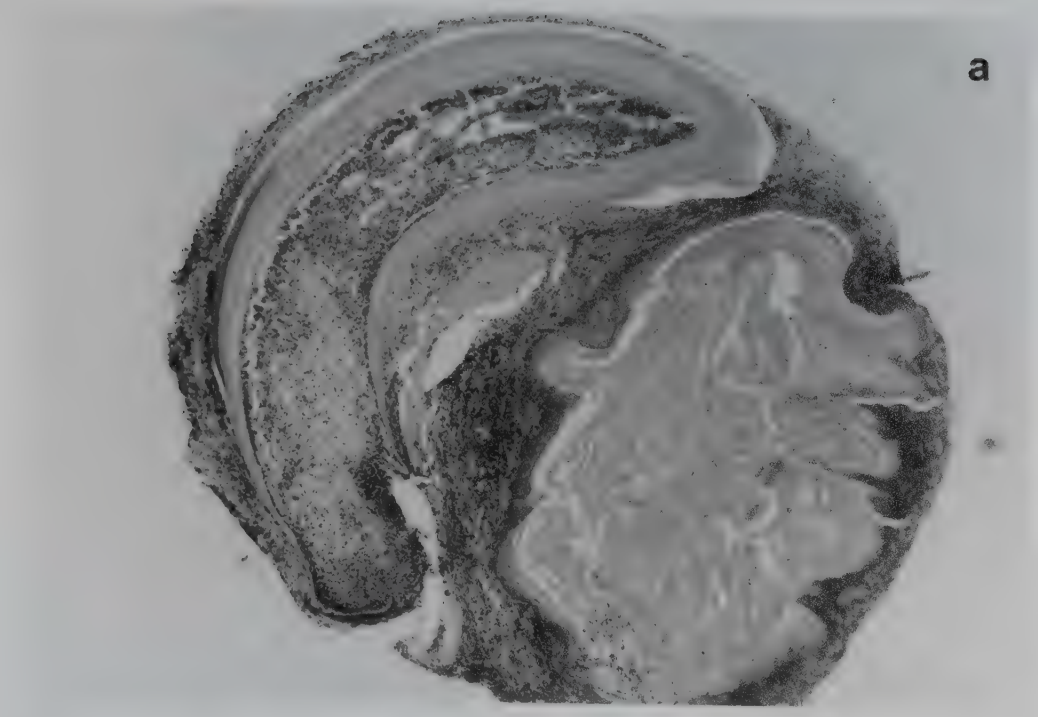

1
2
3

Plate 12

10 day (hematoxylin and eosin stain, magnification x60).

a. Anterior segment graft. An incisor primordium in advanced stage of crown development is present, showing enamel (organic matrix remained after decalcification) and dentin.
b. wax model of primordium featured in a, together with alveolar bone (below) which lay within the concavity of the incisor and separated it from a cyst.


a

B

Plate 13

10 day middle segment graft (hematoxylin and eosin stain, magnification x60)

1. M1 primordium: advanced stage of crown formation.
2. M2 primordium: also showing enamel and dentin formation. The ameloblast layer and odontoblasts are present.
3. M3 primordium: an early stage of development, prior to calcification.
4. sebaceous gland
5. salivary gland tissue
6. bone

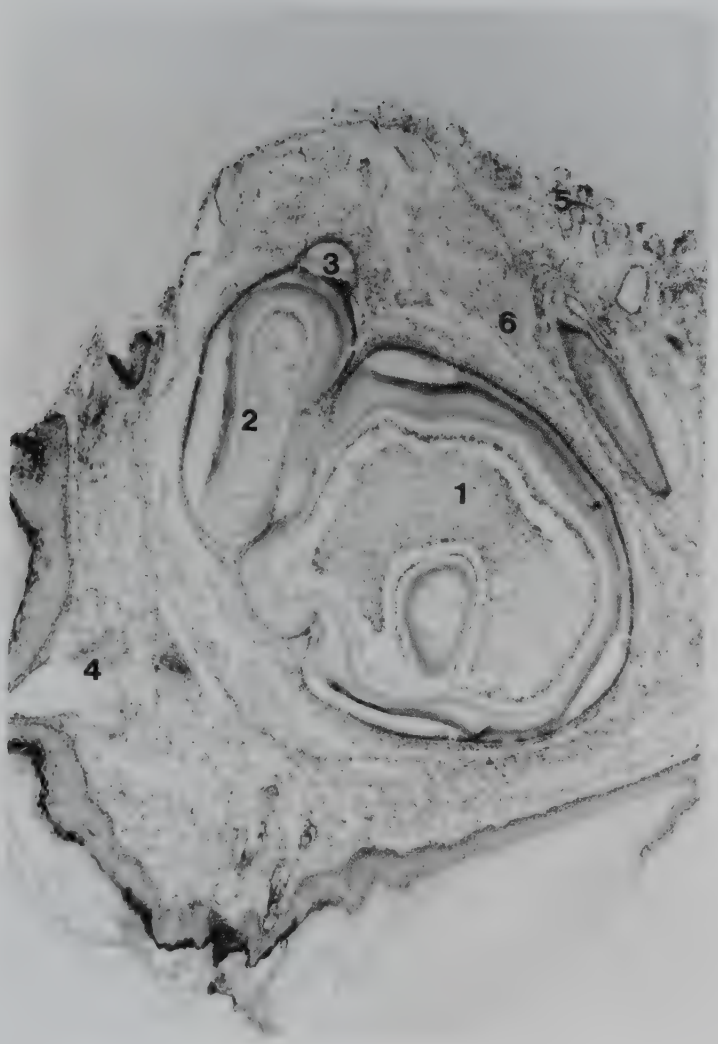

5
3
6
2
1
4

Plate 14

10 day whole arch graft (hematoxylin and eosin, magnification x200)

1. incisor primordium: Enamel is present on only one side and is not folded. Enamel forming cells, the columnar ameloblasts, have differentiated on one side only of the tooth. They can be seen adjacent to the molar primordium (2). The dentine of the tooth is not folded.
2. molar primordium: Enamel is present on all sides and is folded. Ameloblasts are present all around the periphery of the primordium.
3. molar primordium: Organic enamel matrix remaining after decalcification is visible. When enamel is well calcified it appears as an artefact space in histology sections.

3
2
1

Plate 15

11 day anterior segment graft (hematoxylin and eosin stain, magnification x200)
A well formed incisor is present showing both enamel (artefact space) and dentin in advanced crown formation. Columnar ameloblasts are seen only over the apex and left side of this space which has probably been distorted during sectioning. Bone is developing around the tooth.

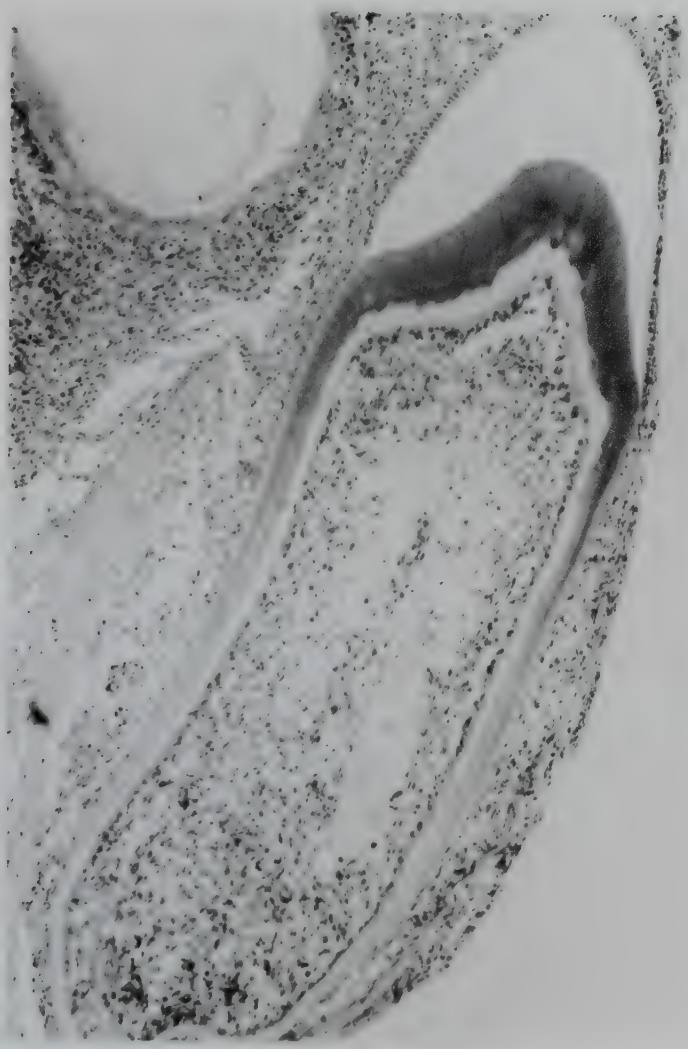


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Plate 16
11 day middle segment graft (hematoxylin and eosin stain, magnification x200)
A molar primordium is present showing enamel and dentin formation as well as the crypt of alveolar bone.

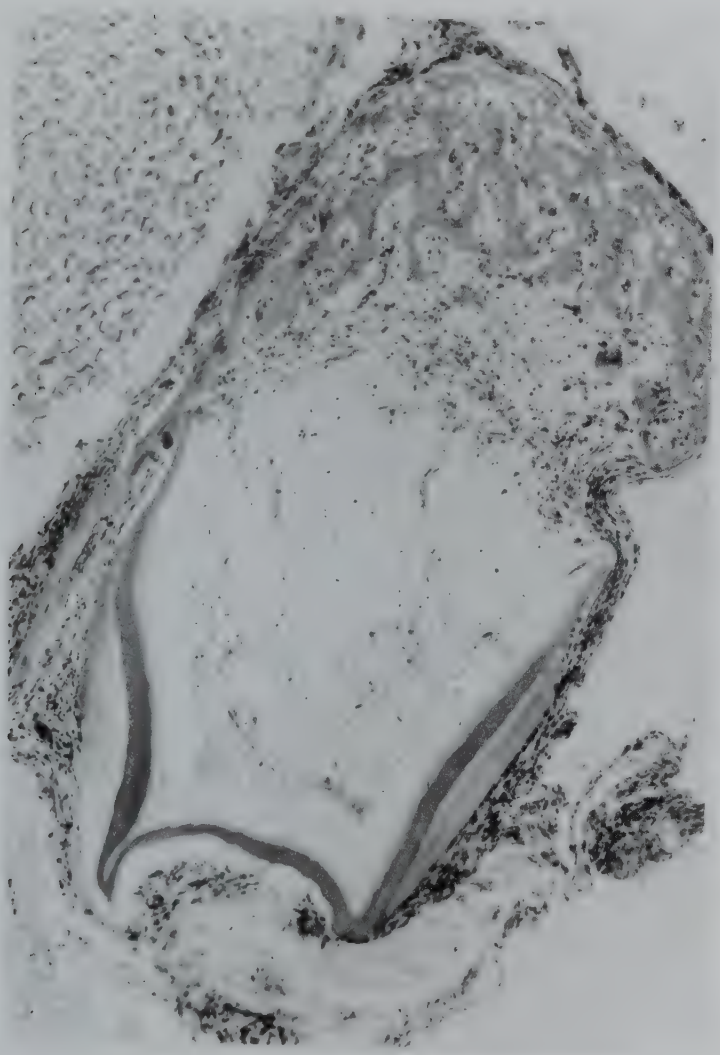
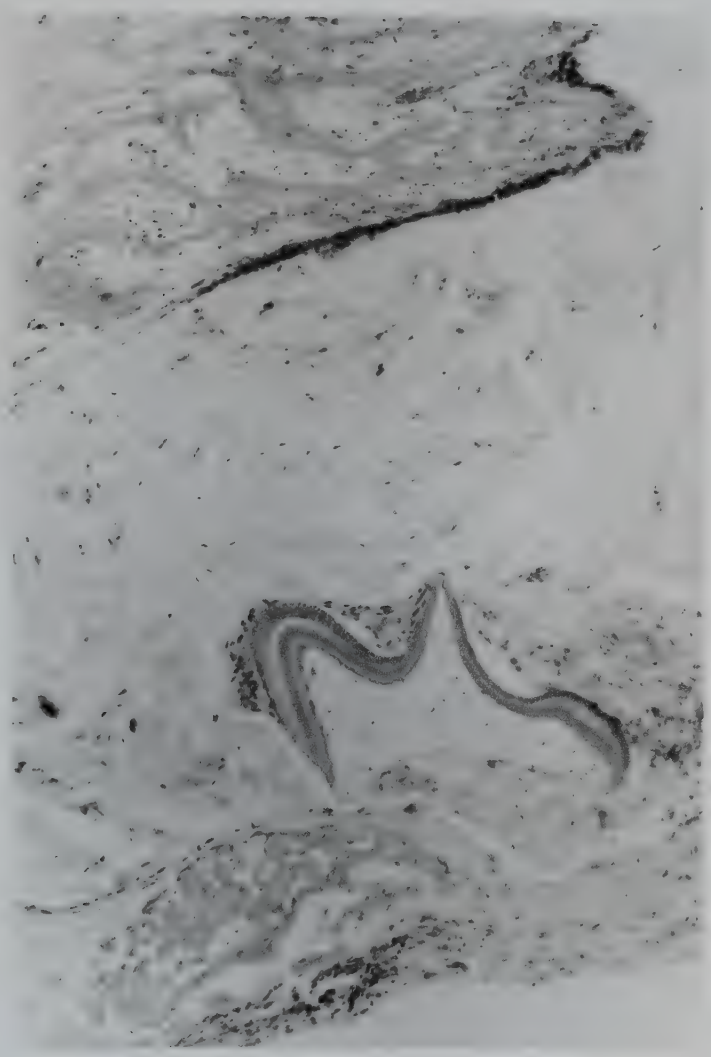


Plate 17

11 day posterior segment graft (hematoxylin and eosin stain, magnification x200)
This primordium was identified as a molar as its enamel surface was folded, however its shape was distorted when compared to a normal molar, perhaps due to the presence of a large cyst in this graft.



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